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C. F. A. PANTIN, Sc.D., F.R.S.

JOHN R. BAKER, D.Sc., F.R.S.

Assisted by

LAURENCE PICKEN, Sc.D.

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Papers should be as concise as possible. Extensive introductory matter and long discussions, commonly required in theses for university degrees, should not be included in the manuscript. Division of the matter by sub-titles must follow a consistent plan. Excessive subdivision must be avoided. Not more than three grades of sub-titles will be printed.

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Figures should be referred to in the text in accordance with these examples: (fig. 3), (fig. 3, D), (fig. 5, D–F), (figs. 3, D; 5, D–F).

The Histology and Activities of the Tube-feet of *Antedon bifida*

By DAVID NICHOLS

(*From the Department of Zoology and Comparative Anatomy, University Museum, Oxford*)

With one plate (fig. 8)

SUMMARY

The histology of the tube-feet and adjacent parts of the water-vascular canal of the crinoid *Antedon bifida* is described. The tube-feet possess the same basic structure as other, better-known tube-feet; here, however, they are adapted to collect food particles. They shoot out mucus by means of special muscle-operated glands and bend rapidly inwards to waft the mucus with entrapped particles into the food-grooves. The protraction of the tube-feet is probably brought about by a mechanism very similar to the ampulla system of other extensile tube-feet, but here the contraction of restricted portions of the water-vascular canal provides the necessary hydrostatic pressure.

THIS paper forms part of a comparative study of the histology, functional morphology, and evolution of the tube-foot system of echinoderms. The purpose of this paper is to describe the histological structure of the tube-feet of the crinoid *Antedon bifida* Pennant and to show that in their basic structure and activities they closely resemble better-known tube-feet, such as those of asteroids (Smith, 1946, 1950) or irregular echinoids (Nichols, 1959 *a*, *b*), despite the impression to the contrary given in many textbooks. Besides the tube-feet themselves the structure of adjacent parts of the water-vascular canal is described, since these are shown to be analogous with the ampullae of other extensile tube-feet, and are thus of importance in protraction.

THE ARRANGEMENT AND ACTIVITIES OF THE TUBE-FEET

The main function of the tube-feet of this crinoid is feeding; they have a subsidiary sensory function. The feeding posture has been described by Chadwick (1907), Moore (1924), and others. When feeding the crinoid sits fastened to the substratum by its cirri and holds its ten arms out almost horizontally with the ends slightly flexed to form a collecting 'bowl' with the mouth at the centre. Each arm bears numerous pinnules on each side which project roughly at right angles. Near the calyx the pinnules of adjacent arms overlap (Clark, 1921, fig. 1044), whereas distally they do not, though the pinnules from adjacent arms nearly touch at their tips. On its oral surface each arm and pinnule bears tube-feet in groups of 3 on each side of a food-groove. On the pinnules the 3 tube-feet of each group are of different lengths and their origins lie in a line at an angle of 20° to 25° to the long axis of the food-grove (fig. 1, B). The

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largest of each group, which are the ones lying nearest the edge of the pinnule, project laterally almost at right angles to the long axis of the pinnule. The tube-feet are set as close together as their movement will allow, so that the food-collecting area available to the animal is practically the area covered by the extended arms: very little of the space within this area is non-effective.

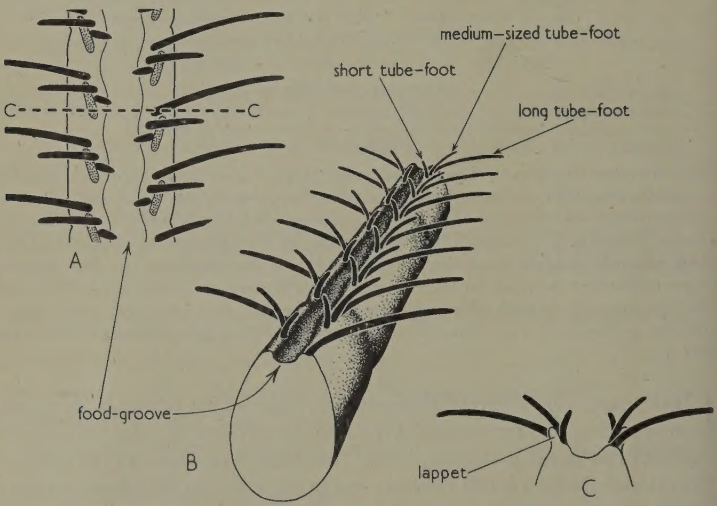

FIG. 1. Diagrams to show the arrangement of the tube-feet on a pinnule. A, plan view of part of a pinnule to show the relationship between the tube-feet (silhouetted) and the lappets (stippled). B, perspective view, looking distally, of part of a pinnule, showing the angle at which the tube-feet are held during feeding. The lappets have been omitted for clarity. C, transverse section across the line *CC* in A, showing the angle at which the tube-feet are held in relation to the lappets during feeding.

The medium-sized tube-feet of each group project upwards and outwards at an angle of about 45° (fig. 1, B, C) while the smallest of each group projects almost straight upwards. Bordering the food-grooves of arms and pinnules are the groove lids or lappets (fig. 1, A, C), which alternate with each other on each side. When the animal is feeding these project upwards at an angle (fig. 1, C), and each long tube-foot projects laterally through the valley between two adjacent lappets (fig. 1, A). The other two tube-feet of each group lie against the inside wall of each lappet with the outer side of the proximal part of the tube-feet fused to this wall (fig. 1. C), so that when the tube-feet bend in towards the mid-line of the food-groove they move the lappet in this direction too. If these tube-feet contract, the lappet closes over the groove, so that only the large tube-feet remain protruding, though usually they too bend inwards to lie across the oral surface of the pinnule.

The tube-feet of the arms are also in groups of 3, except close to the arm bases, where they are single; but they are all of similar size, roughly corresponding to the medium-sized tube-feet of the pinnules, and held at about the same angle. All the tube-feet of *Antedon* are of approximately the same diameter, that is, about 15 to 20 μ. The longest tube-feet of the pinnules extend to about 200 μ; the medium-sized tube-feet of the arms and pinnules extend to about 80 μ, and the short tube-feet of the pinnules extend to about 40 to 50 μ. They can all be retracted to between two-thirds and a half of their extended length.

When the animal is feeding the tube-feet make sudden and rapid bending movements every few seconds. This apparently happens whenever food material touches the tube-feet and stimulates the sensory cells, since crushed fragments of food dropped on to a pinnule cause the tube-feet immediately to bend and flick in an attempt to throw the food into the groove. The rapid movement of the long tube-feet is almost entirely at right angles to and towards the food-groove; that of the medium-sized tube-feet is also almost always at right angles to the groove, but both towards and away from it; the small tube-feet can twitch in any direction. The tube-feet of the arms can move in any direction, though most of their movements are at right angles to the food-groove. After a twitch the tube-feet return to the 'waiting position' more slowly.

MATERIALS AND METHODS

The specimens used in this study were dredged from Millbay Pit, in Plymouth Sound. Fragments of arm, with 3 or 4 pinnules attached, were fixed in Heidenhain's 'Susa' fixative, made up in sea-water, for 6 to 8 hours. Before fixation, some of the animals were narcotized in propylene phenoxytol (Owen, 1955). It was found convenient to pipette 3 or 4 drops of this fluid to the bottom of a 300 ml dish containing the specimens and leave for 24 h, so that the fluid diffused slowly into the water. Specimens treated in this way could thereby be fixed with their tube-feet in a relaxed state; this treatment did not appear to affect the staining qualities of the tissues, which were compared with non-narcotized material. Embedding followed the same procedure as that used for a study of the tube-feet of echinoids (Nichols, 1959 *a*, *b*). For ordinary histological detail Pantin's (1948) modification of Masson's trichrome technique was used, and for special purposes the following additional stains were employed:

1. Heidenhain's iron haematoxylin, for general cytological detail;
2. mucicarmine, muchaematein, thionine and toluidine blue, for mucous glands;
3. Curtis's substitute for van Gieson, for collagen.

THE HISTOLOGY OF THE TUBE-FEET

The stem. The outer epithelium, bounded by a thin cuticle, is continuous with the general epithelium covering the surface of the arm or pinnule. It is one cell thick, except in the region of the papillae (see later), the round or oval nuclei being irregularly grouped in it. In contracted tube-feet, i.e. those from non-narcotized material, the outer surface of the epithelium is sometimes wrinkled in places, but in no case studied was the epithelium thrown into folds. There are no cilia on the external surface. Underlying this layer is the sub-epidermal nerve-plexus, which is continuous with that underlying the

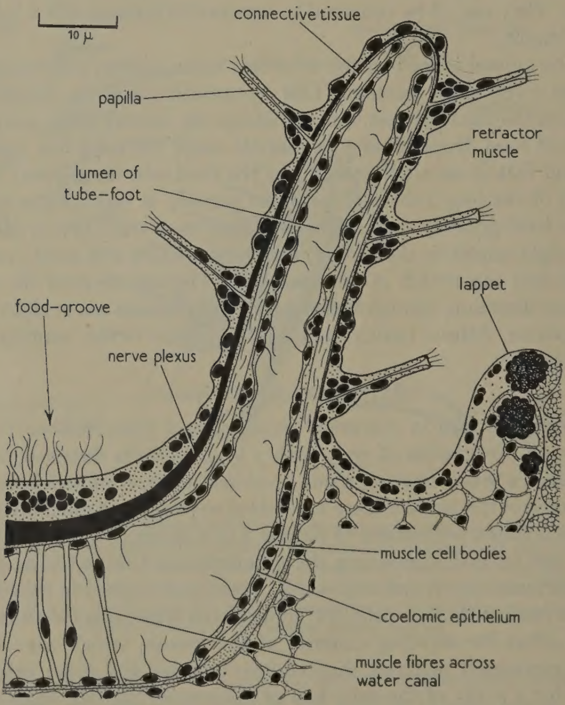

FIG. 2. Longitudinal section of a single large tube-foot of a pinnule, considerably foreshortened for clarity. The end of the lappet, which is not attached to the outer side of the large tube-feet of each group, is shown on the right.

food-groove. It is considerably thicker ($1\frac{1}{2}$ to 2 μ) on the adradial side of the tube-foot (fig. 2) than on the opposite side ($\frac{1}{2}$ to 1 μ), and appears to continue as a thin plexus under the epithelium covering the lappets and the sides of the arms and pinnules and may lead into the lateral brachial nerve. There does not appear to be a space under the epithelium and nerve-plexus, since the degree of extensibility of the tube-foot does not necessitate folding of the epithelium during retraction.

Under the nerve-plexus lies the sheath of connective tissue, the structural framework of the tube-foot. It consists of one layer of circular-running fibres, and does not exceed 2 μ in thickness throughout the length of the stem. The sheath is continuous with that forming the framework of the water-vascular canals, though it is slightly thinner round the canal (1 to 1½ μ). Other series of muscles of the arm or pinnule are attached to it in the canal region (see later).

The retractor muscles form a group or ring, depending on the tube-foot, nternal to the connective-tissue layer. They originate at a swollen region of the connective-tissue sheath proximal to the point where the water-vascular canal divides to form the 3 tube-feet of a group, and insert into the same sheath near the tip of the tube-foot. They do not appear to be attached to the sheath elsewhere down their length. The retractor muscles form a complete ring in only the small tube-feet of each group (figs. 7; 8, F); in the medium-sized tube-feet of the pinnules they form two columns, one on the side facing the food-groove and the other directly opposite; in the large tube-feet there is a single column only, on the side facing the groove. The fibres are between ½ and μ in diameter, those of the small tube-feet being narrower than those of the medium or large. The tube-feet of the arms have a complete ring of retractor muscles. In common with tube-feet of other echinoderms studied in this work there are no nuclei present in the layer of muscle-fibres; the cell-bodies and nuclei of the muscle-cells form a separate layer internal to the layer of fibres. In some cases the cell-walls can be traced centrifugally between the ndividual fibres to their point of insertion on the connective-tissue sheath, hough this is difficult to see.

The innermost layer, lining the lumen of the tube-foot, is a ciliated coelomic epithelium. The delimitation of this layer from the underlying one is not clear, though the cytoplasm of the coelomic epithelium is more granular than hat of the muscle cell-bodies. Also, the nuclei are much more elongated than hose of the muscle-cells.

The papillae. These solid structures appear as nearly perpendicular projections of the external epithelium of all the tube-feet. They average 15 to 0 μ in length and 2 to 3 μ in width except at their swollen bases, where they may be up to 10 μ wide. At the distal end each bears a fringe of 4 to 6 sensory processes. The main structure in each papilla (figs. 3; 8, D) is a single muscle-fibre, about 1 μ thick, which originates at the tube-foot connective-tissue sheath, piercing the sub-epidermal nerve plexus of the tube-foot wall to do so, and inserts into the cuticle at the blunt end of the papilla. The cell-body and nucleus of this muscle are normally found about half-way along its length, hough occasionally they may be nearer the proximal end. A slightly granular cytoplasmic envelope can sometimes be seen surrounding the muscle-fibre for most of its length, and when the granular material is not so conspicuous this sometimes gives the fibre the appearance of being surrounded by a cavity. The swollen base consists of two other types of cell: first, there are from 4 to single-celled mucous glands with long ducts passing up the papilla alongside the muscle-fibre and opening at the tip. These stain well with mucicarmine and muchaematein, and show γ-metachromasia with toluidine blue or thionine. They also take up the light green very faintly in the Masson technique.

Secondly, there are from 4 to 8 nerve-cells, most of which send a single nerv process up the papilla to protrude beyond the distal tip. There are usuall more nerve-cells in the swelling than there are processes at the tip, and it i possible that another sort of nerve-cell, not possessing a projecting process is present. Alternatively, they may provide replacement of damaged processes. From the proximal ends of at least some of the nerve-cells other processes arise which appear to run into the nerve plexus of the tube-foot.

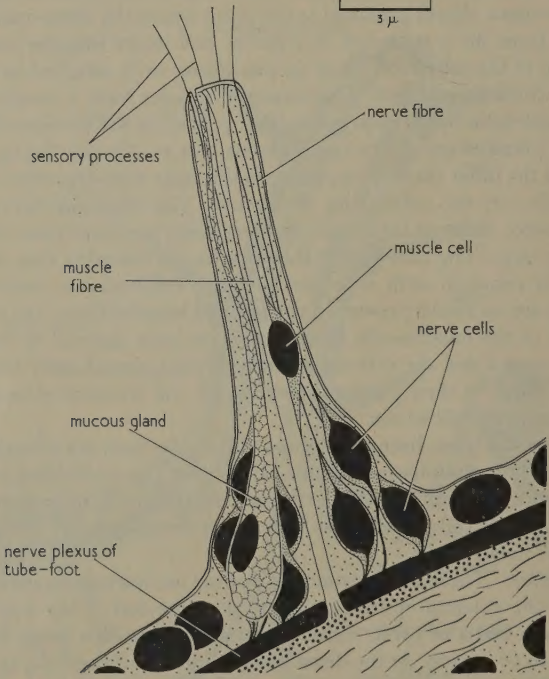

Fig. 3. Longitudinal section of a single tube-foot papilla, showing, on the left, one of the 4 or so mucous glands, and, on the right, 3 of the 8 or so nerve-cells, most of whose processes protrude from the tip of the papilla. The cytoplasmic envelope, which can sometimes be seen surrounding the length of the central muscle-fibre, has been omitted for clarity, except in the region of the nucleus, where it is normally clearly visible.

The water-vascular canal. In the arm of *Antedon* the radial perihaema vessel ('subtentacular canal' of Chadwick, 1907) is double (figs. 4; 8, B), it two elements being separated by a vertical septum containing the radia haemal strand. The oral side of each element of the vessel is a very thin septum, seldom more than 3 or 4 μ thick, which separates the perihaemal syste from the radial branch of the water-vascular system above; it consists of th

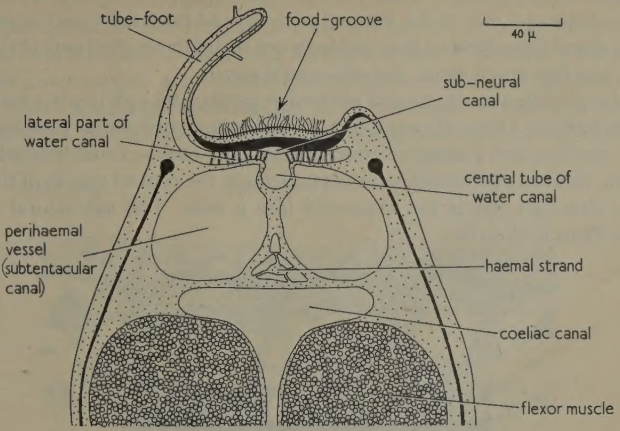
tube-foot
food-groove
40 μ
sub-neural canal
lateral part of water canal
central tube of water canal
perihaemal vessel (subtentacular canal)
haemal strand
coeliac canal
flexor muscle

FIG. 4. Transverse section of the oral part of an arm, with one tube-foot cut in longitudinal section on the left.

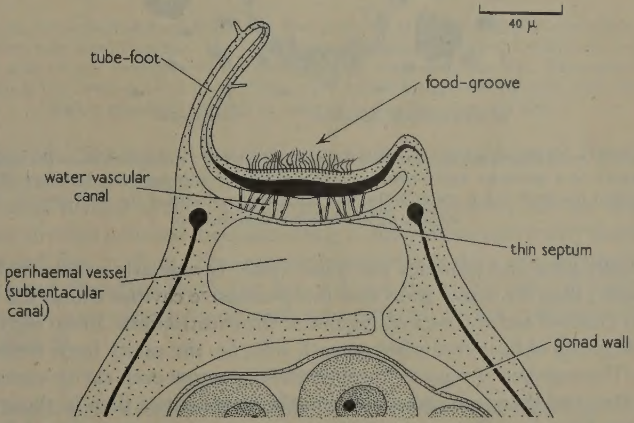

FIG. 5. Diagrammatic transverse section of the oral part of a pinnule, showing one tube-foot in longitudinal section on the left.

coelomic epithelium lining the perihaemal vessel and that lining the water vessel, with a continuation of the tube-foot connective-tissue sheath sandwiched between them. A large number of separate muscle-fibres, about $1\frac{1}{2}$ to 2 μ thick, are stretched across the water vessel, attaching aborally to the connective-tissue layer in the thin septum and orally to the same connective-tissue layer where it passes beneath the radial nerve (see also fig. 2). These muscle-fibres are absent from the centre of the water vessel, i.e. from the region of the oral end of the vertical septum, and here the vessel is expanded into a central tube. Oral to this, and between it and the central part of the radial nerve, is a very small canal, the sub-neural canal.

In the pinnule the situation is somewhat similar, though there is no vertical septum holding a haemal strand (fig. 5), and so the perihaemal vessel is single. There are frequent passages between it and the coeliac canal beneath. As in the arm, there are no muscle-strands traversing the central region of the water vessel, although this is not expanded into a tube. The sub-neural canal is smaller than in the arm.

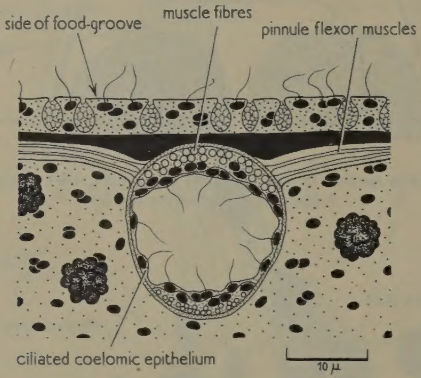

FIG. 6. Semi-diagrammatic horizontal section of part of a pinnule to show the single branch of a pinnular water-vascular canal leading to a group of 3 tube-feet. The section has been taken through the wall bordering one side of the food-groove, the surface of which is towards the top.

In both arms and pinnules the water vessel is regularly constricted along its length; thus the vessel is divided into successive cavities connected by the central channel and the narrow regions at the constrictions. From each cavity a single group of tube-feet arises on each side, i.e. the cavity feeds 6 tube-feet in all. The number of muscle-fibres stretching across each cavity varies from 50 to 100, and there are rather more in the arm cavities than in those of the pinnules. Of course, those muscle-fibres nearest to the constrictions delimiting each cavity are shorter than those at the cavity centre.

The side branches from the water-vascular cavities to the groups of tube-feet (figs. 6; 8, E) have an internal lining of ciliated coelomic epithelium, and, outside this, a sheath of connective tissue, a continuation of the sheaths in the tube-feet. In the pinnules certain muscles are attached to the outer face of this sheath. First, there are two or three fibres running parallel to the long axis of the pinnule and constituting the pinnule flexor muscles. Secondly, there are certain muscles running aborally to the ossicles. It is unlikely that these two sets of fibres have any function directly concerned with the water-vascular system, the connective-tissue sheath merely serving as a convenient anchorage for them. Also attached to the sheath before it branches into the 3 tube-feet, but on the inner surface, are the tube-foot retractor muscles; where they originate the sheath is slightly expanded (fig. 2). In this region of the canal they are in two columns, one on the side facing the food-groove and the other directly opposite to it. The column facing the food-groove contains more fibres than the other, and their diameter is larger.

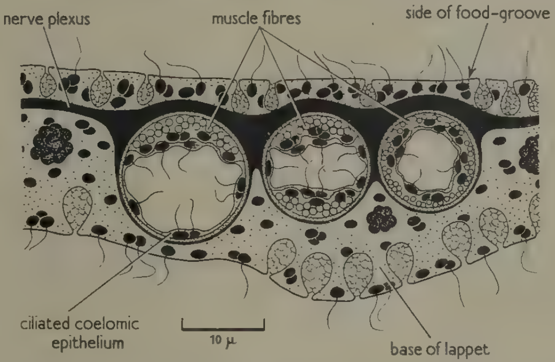

FIG. 7. Semi-diagrammatic horizontal section of part of a pinnule to show the origins of the 3 tube-feet of a group. The section has been taken through the wall bordering one side of the food-groove, the face of which is towards the top. The surface towards the lower part of the picture is the lateral face of the pinnule, with the highly glandular swelling of the base of one lappet on the right.

The canal divides to form the lumina of the 3 tube-feet, and the muscle-fibres are divided between them according to the pattern previously described (p. 109 and figs. 7; 8, F). The nerve-plexus, which does not surround the beginning of the side-branch but lies only on the radial side of it (fig. 6), now sends a thin plexus round each tube external to the connective-tissue sheath (fig. 7).

DISCUSSION

The histological structure of the tube-feet and associated organs of *Antedon* has been inadequately described previously. Ludwig (1877), Hamann (1889), and Chadwick (1907) all give semi-diagrammatic and unsatisfactory pictures of the tube-foot papilla with incorrect interpretation. Reichensperger (1908), however, recognizes the mucous glands and sensory processes of the papilla, and figures a central muscle-fibre, though he considers this to be continuous with the tube-foot retractor musculature. His assumption is that the secretion of the gland-cells is toxic to small animals, and is forced out by the action of the muscle when the sensory processes are stimulated by, for instance, small crustacea. Presumably, with this arrangement, the tube-foot would also retract under the same stimulation, but he does not mention tube-foot retraction; nor does he mention the connective-tissue sheath of the tube-foot, which has been shown in the present paper to separate the papilla muscle from the tube-foot retractor muscles. In no place in the water-vascular system of the arms and pinnules has this sheath been shown to be pierced, although the responses of the tube-feet to stimulation necessitate the assumption that there are nervous connexions across it. Perrier (1886), for the developing *Antedon*, also shows a central filament in the papilla, but describes it as a 'filament tactile' (see, for instance, his fig. 75). He too draws this central filament as leading into and becoming part of his 'couche fibreuse' of the tube-foot wall, and by this it is clear that he means the layer of retractor muscles and not the connective-tissue sheath, which he too does not mention.

Hamann (1889) claims that the epithelium lining the lumen of the water tube consists of epithelio-muscular cells. Presumably he is referring to the tube-feet only, since there are no other longitudinal muscle-fibres lining the water tube other than the tube-foot retractors, which, it is true, originate quite far down the lateral branches of the water canal. In any case, the present work does not support his findings, since the ciliated coelomic epithelium is clearly a separate layer from the layer of muscle-cell bodies (not mentioned by Hamann), in common with the situation in echinoids (Nichols, 1959 *a*, *b*) and asteroids (Heddle, personal communication, 1959).

That the tube-feet serve to trap food seems certain. For this purpose they would require a sensory system, a supply of mucus, and a method of transferring the particles from the 'net' to the food-groove and thence to the mouth. There is little doubt that the papillae of the tube-feet provide the sense cells and a good deal of the mucus, though some more may be provided by the large glands of the lappet walls. The function of the single muscle-fibre in each

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FIG. 8 (plate). A, longitudinal section of a small tube-foot of a pinnule, with two papillae in focus. The section is not quite median, and some of the tube-foot retractor muscles and their nuclei, which apparently form a complete cylinder in this tube-foot, can be seen at lower centre. All sections in this plate are from material narcotized in propylene phenoxytol, fixed in Heidenhain's 'Susa', and stained by Pantin's (1948) modification of Masson's trichrome technique.

B, transverse section of the oral part of an arm, showing the expanded water canal with its tube-like central portion. The central septum separating the two elements of the perihaemal system is much reduced in this section, and the perihaemal canals are flattened dorsoventrally. See also fig. 4.

C, sagittal section of the dorsal part of a pinnule, showing the constrictions in the water canal which delimit the successive hydrostatic cavities supplying the tube-feet. The circular central space without muscle-fibres, to the right of centre, is the origin of a branch of the canal to a group of tube-feet, a continuation of which is shown enlarged in E.

D, transverse section of a large tube-foot of a pinnule, showing two papillae. Note that the tube-foot retractor muscles are in one column only.

E, horizontal section of part of a pinnule, showing the side-branch of the water-vascular canal supplying one group of tube-feet. The edge of the food-groove is towards the top. See also fig. 6.

F, horizontal section of part of a pinnule, slightly oral to that shown in E, showing the origins of the 3 tube-feet of a group. The small tube-foot, on the right, has been cut obliquely, and hence its right wall is out of focus. See also fig. 7.

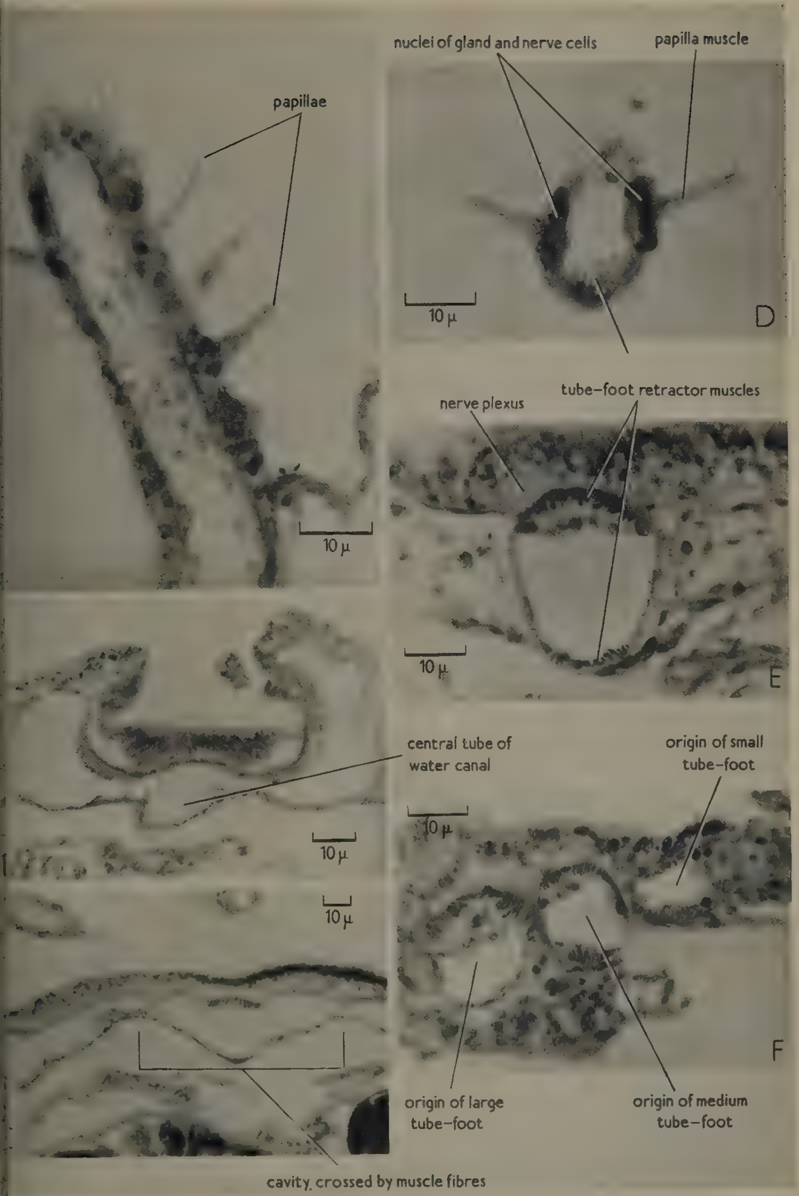
papillae
10 µ
nuclei of gland and nerve cells
papilla muscle
10 µ
D
nerve plexus
tube-foot retractor muscles
10 µ
E
central tube of water canal
10 µ
10 µ
origin of small tube-foot
10 µ
F
origin of large tube-foot
origin of medium tube-foot
cavity crossed by muscle fibres

FIG. 8

D. NICHOLS

papilla is most likely to squeeze out the mucus with some force principally to form the food net when an impulse is received from the sensory processes after stimulation by small crustacea and other food material. In this case, a direct sensory-motor system is suggested, similar to that postulated for the comparable situation in the funnel-building and sub-anal tube-feet of the echinoid *Echinocardium cordatum* (Nichols, 1959*a*), where, as here, mucus is required to be thrown out from the region of the glands rather than to remain covering the epithelium. In both cases the nerve-cells, muscle-cells, and mucous glands are all on the outside of the connective-tissue sheath, and quite separate from the tube-foot retractor muscles.

Direct observation of the feeding method is difficult, but the arrangement of the tube-feet and the nature of their muscles suggests that the main entrapping organs are the large tube-feet of the pinnules and the medium-sized ones of the arms. These will be aided in some measure by the medium-sized feet of the pinnules, though these and the small tube-feet undoubtedly act mainly to transfer the particles into the groove. When the animal is feeding the large tube-feet flick towards the groove, giving the impression of passing the food trapped in mucus to the medium-sized feet and thence to the small ones, and these appear to wipe the food and mucus into the groove. The medium tube-feet are able to move outwards as well as inwards to pick up the food-laden mucous strands, and the small feet can twist in any direction to catch the strands from the medium feet and drop them into the groove. The respective powers of movement are reflected in the arrangement of the musculature of the three tube-feet of a group (see p. 109 and fig. 7). Though the muscles of the medium tube-feet are in two columns and could quite easily be separately innervated, no evidence was found of similar delimitation of the cylinder of retractor muscles of the small tube-feet of the pinnules and the tube-feet of the arms, such as occurs in the echinoid *Echinocyamus pusillus* (Nichols, 1959*b*). Some sort of differential excitation would probably be necessary to explain the erratic flicking of these tube-feet, and this remains a problem for further investigation.

To summarize, the papillae of the tube-feet most probably serve to catch small crustacea and other food material by shooting out a mucous trap. This food is then thrown towards the food-groove by the exceedingly rapid and powerful flicks of the tube-feet. Food caught by the large tube-feet, the principal food-catching organs, is unlikely to be transferred to the groove in one flick and must therefore be assisted by the medium and small tube-feet. It seems likely that a throwing action alone would be unable to detach the mucus-plus-food from any of the tube-feet, and hence the transference is probably aided by forceful discharge of more mucus from the muscle-operated papillal glands at the same time as the foot is flicked towards the groove. Thus, the papillae may have two functions: first, to trap the food, and secondly, to help in food transference.

If an arm or pinnule is stimulated violently the tube-feet quickly retract to about two-thirds or one-half their extended length, and move in towards the groove, closing the lappet over the groove. The return to the feeding posture is much slower, and is indeed reminiscent of ampulla action, such as occurs in asteroid and echinoid extensile tube-feet. Since no ampullae as such are present in *Antedon*, it is likely that their function is performed by the expanded water-vascular canals, which are constricted into successive cavities (fig. 9) and provided with traversing muscle-fibres. It seems probable that protraction of the tube-feet of a group is brought about by the closure of *first*, the canal constrictions which lie across the long axis of the arm or pinnule, and *secondly*, the border of the central tube-like part of the water-vascular canal on the appropriate side (fig. 9, B). Further contraction of the traversing muscle-fibres, particularly those towards the centre of the cavity now cut off, would force water into the tube-feet of that side and cause their protraction.

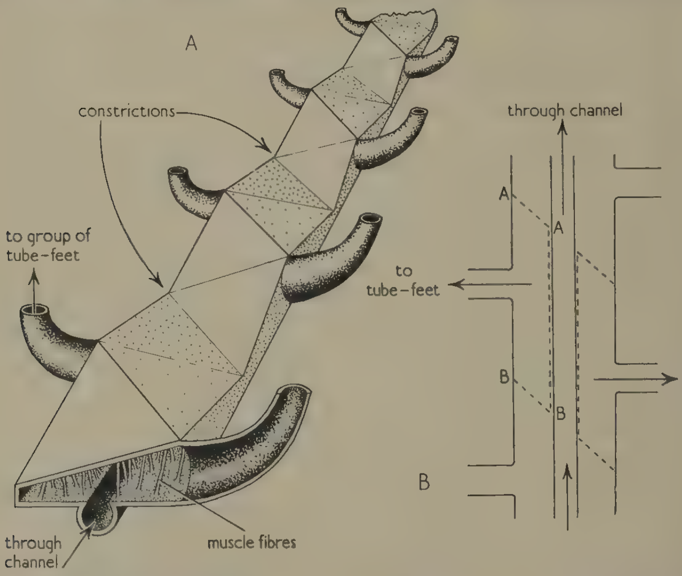

FIG 9. Schematic diagrams to show the structure and suggested mode of action of the water-vascular canal. A, perspective view of the canal of an arm, showing the relationship between the constrictions, which cross the canal diagonally, and the side-branches to the tube-feet; the canal is highest where the side-branches arise. It is traversed internally by muscle-fibres, except in the central tube-like portion, which provides a through-channel to distal regions. In the pinnules the situation is similar, except that the central through-channel is less pronounced. B, plan view of the canal of an arm or pinnule, showing the suggested mode of protraction of the tube-feet. The dotted lines show the supposed delimitations of one cavity on each side. Considering the left-hand cavity only, contraction of the muscle-fibres at the diagonal constrictions *AA* and *BB* and of those lining one side, *AB*, of the through-channel, isolates a section of the water canal; further contraction of muscle-fibres within the compartment thus formed forces water out through the side-branch to the three tube-feet while still allowing fluid to pass down the central channel.

During this process it will be necessary to maintain a flow of water to the portions distal to the protracting tube-feet, particularly in the arms, since the pinnules distal to the active tube-feet will be dependent on the supply getting past the region of activity; the central tube-like part of the water canal in each arm presumably provides a passage for this flow. In a pinnule, a through-passage may not be so important, there being less distal dependence on the free flow of water, and it may be because of this that the central part is less clearly delimited.

By this arrangement it can be seen that each group of tube-feet will be supplied by its own hydraulic system, and all 3 tube-feet of the group will necessarily be protracted together; this is supported by observation of the active animal. Each tube-foot appears to be able to retract independently, however, which suggests separate innervation for each tube-foot, probably originating at the sensory processes of the papillae. There is also likely to be a 'central' innervation of the musculature of each group by way of the main radial nerve; for instance, for rapid closure of the food-grooves.

It is a pleasure to record my indebtedness to the Director and Staff of the Marine Biological Laboratory at Plymouth for help in obtaining material, and for providing facilities for some of the work; to Mr. Duncan Heddle for useful discussion; and to Prof. Sir Alister Hardy, F.R.S., in whose laboratory most of the work was carried out.

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The Structure of the Nervous System in *Velella*

By G. O. MACKIE

(*From the Department of Zoology, University of Alberta, Edmonton, Alberta, Canada*)

With two plates (figs. 2 and 4)

SUMMARY

Silver staining methods have been applied to the nervous system of *Velella*. Two istologically distinct plexuses are described under the headings ‘open’ and ‘closed’ stems. The open system consists of neurones with fine processes which run for istances of up to 2 mm, retaining their independence ins pite of frequent contacts ith other fibres. The fibres of the closed system are large and run together, forming nerve-net in which neurofibrillar material from different neurones intermingles; it is rovisionally to be regarded as a syncytium. A certain type of ‘fibre’ in this system believed to arise secondarily by the drawing out of adhesion connexions into long rands. Free nerve-endings resembling growing-points occur in both systems. The vo systems occur throughout the ectoderm, but in the invaginated ectoderm the open stem is poorly developed. The functions of the two systems are not known, but the osed system is probably specialized for through-conduction.

Neuro-sensory cells occur in the external ectoderm, making contact with fibres of oth open and closed systems. No specialized endings have been found in a muscular gion examined. No nerve-rings or centres have been found. Nerves are sparsely istributed in the endoderm, but they lie independently of one another and of ectoermal nerve-fibres crossing the mesogloea between the invaginated and external toderm layers.

INTRODUCTION

THE nervous system of *Velella* was discovered by Chun (1881, 1882). Conn and Beyer (1883) were the first to investigate the nerves of the related rm *Porpita*. Of subsequent work, reviewed by Chun (1902), Schneider’s inestigation (1892) is of particular importance. As Chun observes, ‘*Velella* rovides brilliant demonstration material for study of the coelenterate nervous stem’. In view of this, and since no further work has been done on *Velella* uring the last 60 years, a reinvestigation with modern staining methods was ndertaken, the results of which form the subject of this paper.

The confusion surrounding siphonophore morphology and taxonomic relaons has largely been dispelled during the last thirty years. It is clear from e studies of Leloup (1929) and Garstang (1946) that the Chondrophora *orpita*, *Velella*, and *Porpema*) are not medusoid organisms in any sense but e really highly modified, free-living hydranths that can be homologized ith sessile tubulariid hydroids such as *Corymorpha*. I have discussed this greater length elsewhere (Mackie, 1959) and have endeavoured to trace ehavioural as well as morphological homologies between *Porpita* and *Coryorpha*. Fig. 1 shows the correct relationships. The chondrophores are distinct om, or only very distantly related to, the true siphonophores. In the most

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authoritative recent account, Totton (1954) ranks the group as a separat order from the Siphonophora.

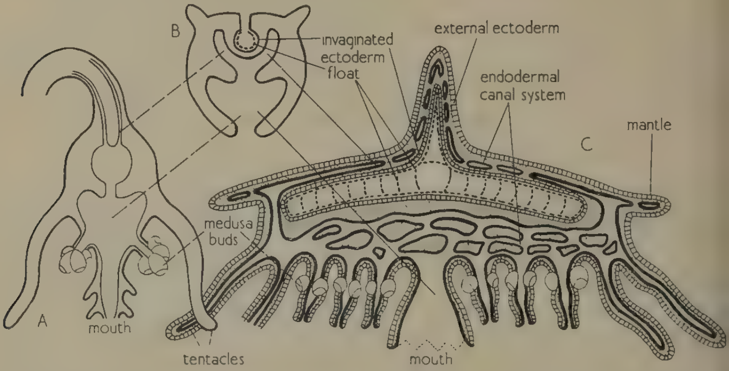

FIG. 1. Diagrammatic sections comparing a hydroid polyp such as *Corymorpha* (A) with th young and adult stages of *Velella* (B and C). Broken lines connect the corresponding parts o A with those of B or C. Continuous lines connect the parts of B with what they become in c Redrawn with modifications from Hardy (1956).

MATERIAL

According to Totton (1954) there is probably only one species of *Velella* the cosmopolitan *V. velella* L. The specimens of *Velella* used in this investigation were obtained from Naples, Villefranche-sur-Mer, Arrecife (Canar Islands), Smitwinkel's Bay (Cape Province, Union of South Africa), an Long Beach, Vancouver Island. The bulk of the material used came from the South African collection, which was kindly sent me by Dr. N. A. H. Millard The specimens chosen for examination were about an inch long. *Velella* grow to over 2½ in. in length. The fixative used was Bouin made up in sea-water.

METHODS

While the general distribution of nerves in a coelenterate can be made ou with the aid of simple dyes or by use of the phase-contrast microscope investigation of their fine structure requires special techniques. Of these methylene-blue staining and osmic-acetic maceration have been most used i the past. Metallic impregnations have been less employed. However, silve methods are now available which give repeatable and consistent results, an have the further advantage over methylene-blue and maceration technique that they start with properly fixed material and end with permanent preparations. Methylene blue rarely stains all nerve-elements at the same time an is hard to make permanent. The results of maceration methods are often ope to doubt because of the possibility that degenerative changes have occurre in the cells. Even among specialists of the osmic-acetic technique there i some lack of agreement. Krasińska (1914), for instance, casts doubt on the value of Schneider's results (1892), saying that he overmacerated or macerated badly. Schneider's procedure is, however, still followed (Semal-Van Gansen, 1952). While it is true that methylene blue and osmic-acetic maceration are of value for special purposes, it is becoming clear that silver methods are generally to be preferred.

The procedures used in this investigation were based on Holmes's buffered impregnating solution. This was followed by hydroquinone/sulphite reduction and gold toning as in Holmes's original method (1947) or by development in Peters's physical developer, which contains glycin (Peters, 1955). Both paraffin sections and thin strips of tissue treated whole were employed. The latter proved most useful although they were harder to stain evenly. A pH of 8·0 was found to be suitable for the impregnating solution. Experiments with temperature, dilution, pH, and concentration of silver ions in the physical developer were crucial in obtaining good results. Satisfactory development was obtained with a modified Peters solution made as follows:

sodium citrate, 0·1 M, 10 ml
Peters's developer, stock solution, 10 ml
distilled water, 25 ml.

To the above add 0·1 M citric acid until a pH of 6·3 is reached. To 45 ml of the mixture add 12 drops (0·7 ml) of 1% $AgNO_3$. Develop at 15° C. The physical developer gave a stronger, more contrasty picture of the nerves than did hydroquinone/sulphite reduction, but it was hard to get really fine-grain development by this means, although many attempts were made with different mixtures, temperatures, &c.

Interpretation of silver preparations

It is true, as Pantin (1952) states, that structures other than nerves are often picked out by silver methods. Among the structures stained most sharply are flagella, discharged nematocyst filaments, centrioles, the membranes of nuclei, nucleoli, and cells, spindle fibres, and fusomal structures (mitotic spindle relics). The latter are frequently found connecting young cnidoblasts, as I have described for *Physalia* (Totton and Mackie, 1960), and it is possible that some authors may have mistaken them for nerves. On the whole, however, if the preparation is a good one and the pH of the impregnating solution has been correctly adjusted, there is never any risk of confusing these structures with nerves. Muscle-fibres usually stain slightly at pH 8·0, but they are unlikely to cause confusion even in the worst preparations. Filamentous, branching processes running from the endoderm into the mesogloea are common in *Velella*, as in many Hydrozoa, but their general topographical relations distinguish them from nerves. In hydrozoan medusae, strongly argentophil branching fibres occur in the mesogloea and these might cause confusion were it not for the fact that they lack nuclei. The mesogloea in *Velella* is completely unstained by silver methods. Dr. K. M. Rudall has shown that it gives the X-ray diffraction pattern of a collagen.

It is probably true that there is little risk of mis-identification of structures in silver preparations provided that the investigator is aware that other structures may stain and further attends to the pH of impregnation. Peters (1955) gives an interesting table showing how depth of staining in nerve-fibres, nuclei, cytoplasm, and connective tissue may vary with the pH.

Distribution of the nerves

Nerves are found in the ectoderm and mesogloea and around the endoderm canals. The nerves crossing the mesogloea run between the external ectoderm and the internal, invaginated ectoderm (fig. 1) which secretes the chitinous float material. In crossing the mesogloea they do not associate with the endoderm canals as far as can be seen but pass between them without making contact. The nerves described here as endodermal are very sparsely distributed and lie by themselves, without connexions with one another or with the ectodermal fibres crossing the mesogloea. The few examples seen have all been closely wound around the canals, and are thus distinguished from the fibres which cross the mesogloea uniting the ectodermal plexuses. The endoderm of the organs of ingestion has not been examined. Fig. 4, A shows the cell-body (*cb*) of a neurone lying in the mesogloea. Several nerve-fibres (*nf*) are shown. Filamentous endodermal processes (*fep*) are also seen. Flagella (*f*) project into the lumen of the canals (*c*). Symbiotic algae (*zx*) are found in many parts of the endoderm.

Chun (1881) states that there are no organized tracts or centres in the nervous system. This appears to be true. Later, however, (1902) he refers to a special region of the plexus in the mantle which he believes to be homologous with one of the marginal nerve-rings of medusae. I have examined this region carefully and find that the plexus is well developed but that it is not organized into anything comparable to the tight bundle of fibres comprising a medusoid marginal nerve-ring. On many other grounds we now know that attempts to homologize *Velella* with a medusa are valueless.

The nerves in the tentacles, reproductive appendages, and hypostome have not been studied closely, but their existence has been confirmed.

Components of the ectodermal nervous system

There are two histologically distinct systems of nerve-fibres and, in addition, neuro-sensory cells are present. The two systems of nerve-fibres will be described under the headings 'closed' and 'open' systems. Fig. 3 shows a portion of the nervous system in which all components are well represented.

The closed system. The fibres of this sytem are the most conspicuous nerve-elements in the animal, and are the elements dealt with by the earlier authors. The word 'closed' is used here because the processes constituting the system run together in net-like configurations. The nerves running in the mesogloea

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FIG. 2. (plate). Closed system. In A the arrows trace a continuous pathway between neurones B and G. The points labelled B to G are shown at higher magnification in the insets on the left.

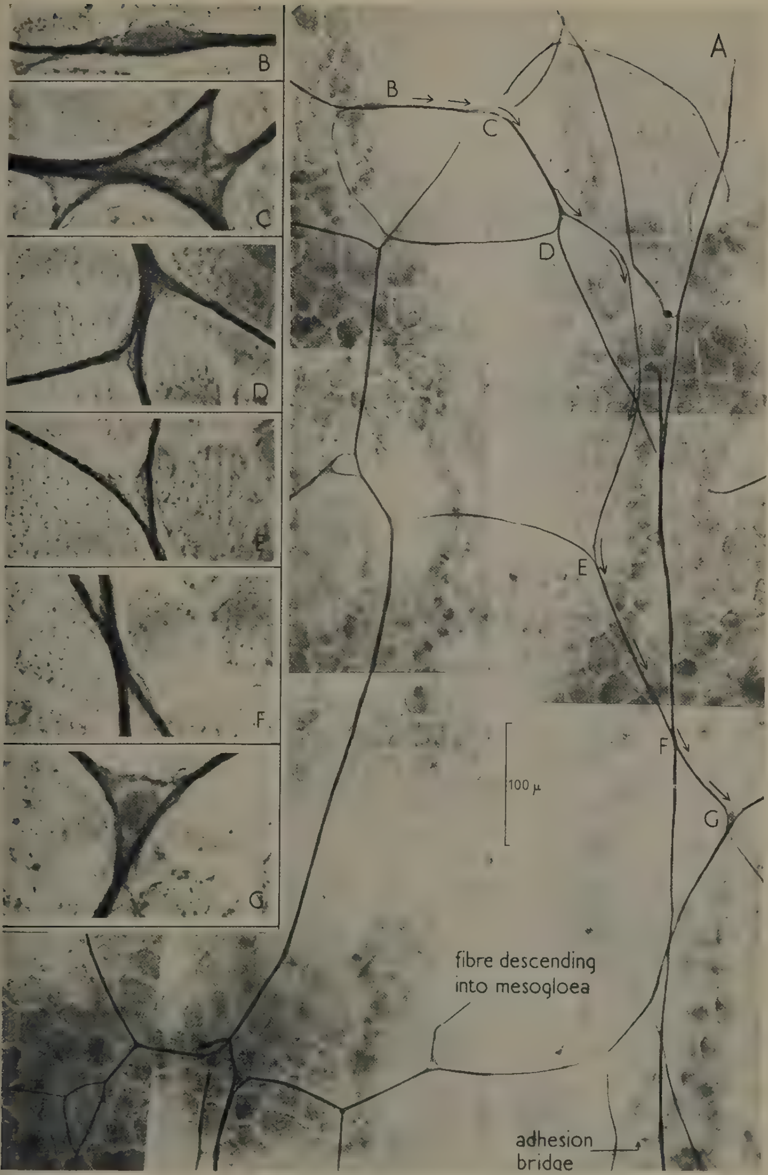

FIG. 2

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and in the invaginated ectoderm nearly all belong to this system. In the external regions the open system of fibres is equally well or even better developed, and complicates the picture. For this reason, the fine structure of the closed system is best studied in the invaginated regions.

The main features of this system are already known from the descriptions of Chun and Schneider. Chun states that the neurones are mostly 3- or -polar but that 2- and multi-polar examples occur; that the fibres branch requently and run in straight lines or strong curves for long distances; and hat at dichotomies and intersections a *dreieckige Verbindungsplatte* (referred o as a triangular veil in the present paper) is often found. Schneider (1892, 902) describes details of the neurofibrillar system in *Velella*. He finds that he finest neurofibrils (*Elementarfibrillen*) run throughout the nerve-cell body nd processes, forming a grid or reticulum (*Zellgitter*) in the cell-body, and un directly from cell to cell forming an intercellular grid (*Elementargitter*). ome of these elementary fibrils run across the nerve-net through side ranches, avoiding the cell-bodies.

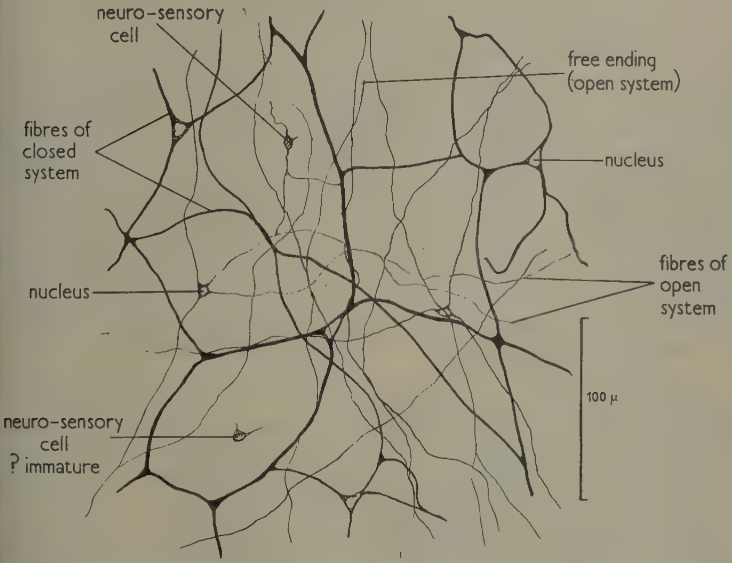

FIG. 3. A characteristic portion of the nervous system from the external ectoderm. Based on a camera lucida drawing.

The fibres of the closed system vary in diameter between 1 μ and 5 μ. The ibrillar material is usually clumped into a darkly staining axis around which he cell-membrane can be made out as a thin crumpled structure (figs. 2, F; , E; 5); in certain places, however, the fibrillar material can be seen to be pread out across the whole breadth of the fibre (fig. 4, F, which is another region of the same process seen in fig. 4, E; both are in the mesogloea). The triangular veils formed at dichotomies (e.g. figs. 2, E; 4, L) appear to be folds of the cell-membrane drawn out between the dividing branches. The fibrillar material at dichotomies usually divides equally, the point of divergence of the two streams of fibrils being quite precise. The fibrils are visible in the cell-body, passing around the nucleus either symmetrically or not, depending on the relative thicknesses of the processes (fig. 4, L). Points of contact in the closed system present a variety of appearances (figs. 2, C–F; 4, J, M, O). Frequently membranous veils occur, uniting the associating fibres. Usually such junctions appear continuous as far as the membranes are concerned, and frequently fibrillar streams can be seen crossing the junction and mingling with the fibrils of other processes (fig. 4, J, M). Other junctions may be discontinuous, there being no apparent mingling of fibrils or evidence of membrane continuity. The question of these neurofibrils and their interrelations will be discussed below (p. 127). The concept of the synapse is hard to relate to this material because even where the fibrils of associating processes appear to remain independent (fig. 4, O), there may be no trace of discontinuity between the membranous parts of the processes. It is perhaps best to reserve the term for physiological purposes.

In many regions it is possible to trace a pathway from one neurone to another through a series of direct connexions in which there appears to be actual fibrillar continuity. This accords with Schneider's findings. Such a pathway (shown by arrows) is plotted out in fig. 2, A, certain of the regions being shown enlarged (fig. 2, insets B–G). Fig. 2, A also shows two neurones in the bottom left-hand region which are probably in direct connexion with each other and, by different pathways, with G and thence with B. At F an intersection probably of a discontinuous character occurs with a fibre not involved in the direct pathway under consideration.

It will be seen from this figure that fibres associating with one another frequently lose their identity, or appear to do so. Only rarely do two fibres associate, run together side by side for some distance, and then diverge again, as in the open system. The result is a nerve-net in which it is impossible to

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FIG. 4 (plate). A, nerves crossing the mesogloea (explanation in text, p. 122).

B, free ending of a closed-system fibre in the mesogloea.

C, free ending of an open-system fibre.

D, portion of the nervous system in the mantle ectoderm. Compare fig. 3.

E, closed-system fibre in mesogloea, showing fibrils clumped.

F, the same fibre as in E in another region, showing fibrils spread out.

G, adhesion bridge in the closed system.

H, portion of the nervous system from the invaginated ectoderm showing a fine fibre of the open system (*o*) and some freely ending branches of the closed system.

J, multiple interconnexion in the closed system.

K, teased ectoderm, showing epithelial cells and a damaged neuro-sensory cell.

L, cell-body of neurone in the closed system.

M, interconnexion in the closed system.

N, free nerve-ending from the invaginated ectoderm.

O, double junction between two fibres of the closed system.

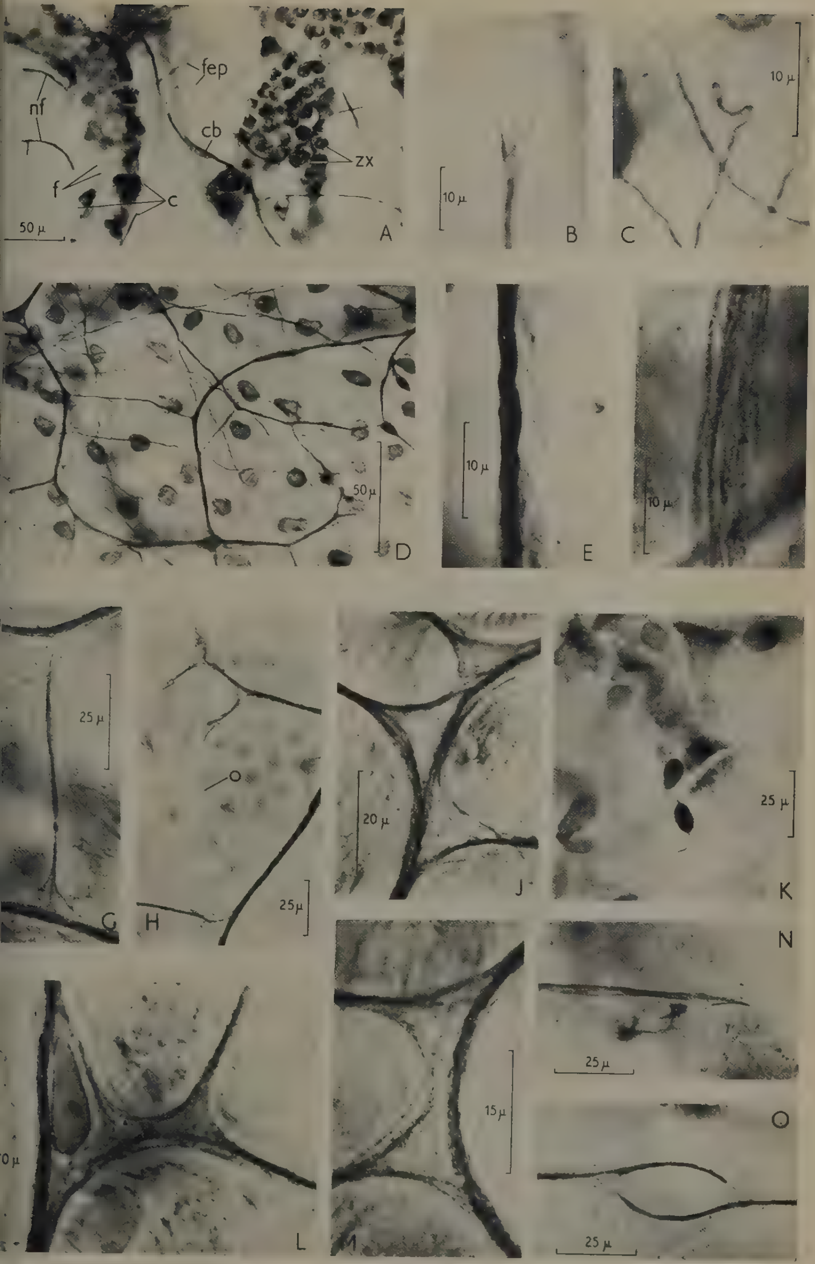

Fig. 4

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say where a fibre ends. Although fig. 2, E is here called a dichotomy of a process originating from neurone G, it might be a junction between this process and one from the left, the latter originating elsewhere. The general shape of the structure and the relative thicknesses of various fibres in the locality, however, make this rather unlikely.

Connexions of a certain type, here called 'adhesion bridges', appear to form a distinct category in the closed system (figs. 2; 4, G). My interpretation of these structures is shown schematically in fig. 5. It is suggested that they arise by adhesion of the membranes of two processes during growth and that the processes are subsequently carried apart by general tissue growth, the adhesion bridge being pulled out into a thin strand. All conditions from short, broad webs to strands 200 μ long and less than ½ μ thick are found. In some of these, fibrillar material appears to have been drawn out into the strand or to be growing out into it, but usually the bridges seem to be empty of fibrils. In fig. 4, G what appear to be fibrils in the veil region are probably creases in which silver has been precipitated, or lines of stress. However, here and there in the nerve-net transverse connexions are found which might represent adhesion bridges in which fibrillar continuity had been established.

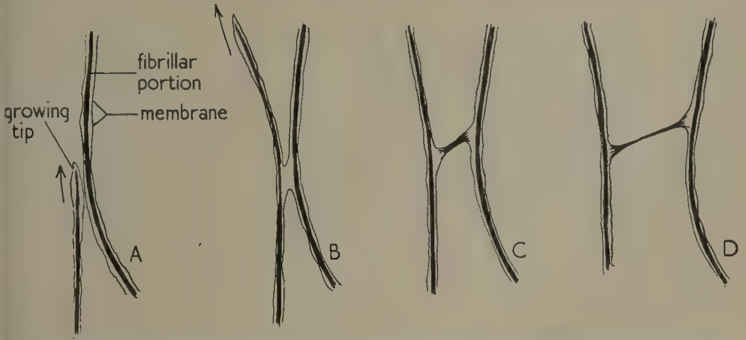

FIG. 5. Suggested origin of adhesion bridges. In A an advancing fibre makes contact with another fibre. In B the cell-membranes are shown forming a connecting web. In C and D general tissue growth carries the fibres apart, stretching out the adhesion bridge.

Free endings in the closed system are either single (fig. 4, N), or branched (fig. 4, H). The free extremity is membranous and without fibrils. Free endings have been found in the mesogloea (fig. 4, B), which resemble growing tips. It is possible that all free endings in the system are growing points. No muscle fibres occur in the regions shown in the photographs.

The open system. The fibres of this system do not form a net but run ndependently throughout their courses despite frequent close juxtapositions with fibres of both systems. The fibre diameters in this system are ¼ to ½ μ. It is easy to distinguish them from the majority of closed-system fibres on this basis alone. They can be distinguished from adhesion bridges, which approach them in diameter, by their discontinuous associations.

The open system is well developed in all external regions examined but is very sparsely developed in the invaginated parts. A fibre of the open system is shown in fig. 4, H (*o*). Sometimes the fibres in the invaginated region run at large in this way, but more often they cling to the closed-system fibres, twining around them like beans around a pole. Some of these fibres have been traced for distances between 1·5 and 2·0 mm. In the external ectoderm (fig. 4, D) the fibres show less tendency to twine around closed-system fibres, possibly because the layer is less flattened and they have more freedom of movement during growth. It is of interest that in the ectoderm below the disk, where nematocyst production adds greatly to the thickness of the layer, the fibres of both systems run freely, making few contacts with each other. The types of connexions found, however, are similar to those found elsewhere. The cell-bodies in the open system contain less cytoplasm than do those in the closed, and details of neurofibrillae have not been made out. The nuclei in the two systems are of comparable dimensions.

Free endings in the open system usually have a bladder-like form (figs. 3; 4, C). What such structures represent is discussed below (p. 127).

Neurosensory cells. Cells of this type occupy a sub-epithelial position, but do not lie as deep as the nerve-fibres. They have conical projections running up to the surface of the epithelium, from which sense hairs project. The latter are thick and short, measuring 2 to 6 μ. They colour strongly in silver preparations. The conical eminence contains granular material but there are no special structures associated with the base of the hair. A teased neuro-sensory cell is shown lying on its side in fig. 4, K. Some teased epithelial cells are also shown and it will be seen that these are wider at the free end than at the base in which the nucleus lies. The nerve-fibres run in the spaces between these narrow stems in the intact tissue. The free ends of the epithelial cells join one another, giving polygonal patterns when seen in surface view (fig. 2, A). The teased preparation (fig. 4, K) was made by stroking the surface of the tissue strip with a blunt needle just before covering with the coverslip. It will be seen that part of the fibre at the base of the neuro-sensory cell has been broken off by this procedure.

Other neuro-sensory cells are shown in fig. 3. The number of processes varies from one to five. Those with three seem most typical. The processes are generally shorter than 100 μ but some have been found as long as 150 μ. In diameter the processes compare with open-system fibres (measuring $\frac{1}{4}$ to $\frac{1}{2}$ μ), but unlike the latter they are often thicker and more densely staining near the cell-body than at their extremities. The processes either end freely or make contact with fibres of the open system or with those of the closed. In one preparation from the mantle region, of 28 processes examined, 6 ended on the open system and 14 on the closed, while 8 ended freely or could not be traced to their conclusions for some reason. In all cases where the junctions were clearly seen, they appeared to be of the discontinuous type. Many of the free endings are swellings, similar to those seen at the ends of the open-system fibres. Other endings are thin tapering structures. Here and there neuro-sensory cells with very short processes are found; these might be confused with interstitial cells (which are also present) were it not for their sense hairs. It is possible that these cells are undergoing differentiation into neuro-sensory cells.

With regard to distribution, neuro-sensory cells are present in all external regions examined, but are absent from the invaginated regions.

Neuro-muscular relationships

Ectodermal muscle in the adult *Velella* is confined to the crest which surmounts the float, to the mantle region and to the tentacles, reproductive appendages and hypostome ('gastrozooid'). In the mantle region nerves run all over the muscle-layer and I have examined them for possible specializations. Contacts between nerves and muscle-fibres appear to be undifferentiated. No special endings or elaborations have been found. Other regions have not been closely examined.

Possible artifacts

In the closed system, as explained, fibrils are visible in the cell-bodies, at junctions and dichotomies, and often in the fibres themselves where they may be spread out or clumped into dense strands. Electron microscope studies of nerve structure in higher animals show that neurofibrils are genuine structures and are not fixation artifacts. However, it is not clear whether the fibrils seen in Bouin-fixed *Velella* nerve are actual neurofibrils, bundles of small, visible elementary fibrils (as Schneider maintains), or groups of submicroscopic fibrils aggregated by fixation. The methods employed in this investigation show the distribution of fibrillar material but do not give a very precise picture of the fibrils themselves and their interconnexions. From Schneider's illustrations it would appear that he was able to make out far more detail by maceration methods. Possibly silver staining applied to material fixed in a mercuric chloride fixative would show the details more clearly.

The triangular veils occurring at dichotomies and intersections are also described in the early accounts; similar veils occur in *Porpita* material fixed in Baker's formaldehyde-calcium made isotonic with the sea-water, and comparable structures are found in a number of other nervous systems, e.g. the atrial nervous system of amphioxus (Boeke, 1935; Bone, 1958). It is unlikely that they are artifacts.

The bladders at the tips of the free endings of neuro-sensory cells and in the open system resemble growing points in frog neuroblasts described by Harrison (fig. 10, C in Kappers, Huber, and Crosby, 1936). However, McConnell (1932) actually observed such bladders forming at free nerve-endings in *Hydra* as an artifact during methylene-blue staining.

Beading or vesiculation of nerve-fibres is usually believed to be a degeneration artifact, although according to Meyer (1955) it may occur in normal healthy nerve-fibres as well as in damaged ones. Beading is occasionally seen in *Velella* nerve-fibres.

In some preparations which were probably stretched or pinched during dissection densely staining *striae* occur in the substance of the fibres and at junctions. These evidently represent slight tears or folds in or on which the silver has collected. The nerves are probably the least elastic structures in the fixed-tissue strips and they frequently show damage where other structures are intact.

Contact and continuity

Work with the electron microscope over the last decade has made it appear unlikely that there is continuity of neurones in those parts of the central nervous system or retina of higher vertebrates where cases of continuity had previously been believed by many to exist (Bielschowsky, 1928). The nature of the interconnexions in the gut plexuses is, however, still in doubt. Reiser (1959) says: 'It is perhaps still possible that nervous tissue is not built according to one single principle but combines within it two regionally differentiated structural planes, the neuronal and the syncytial.'

In the case of Invertebrata the same holds true, but the evidence for the syncytial type of neurone system, particularly in certain giant-fibre systems, is very much stronger. Young (1939) has given the clearest possible evidence for the syncytial character of the giant fibres in *Loligo*. Both in annelids and in arthropods the fusion of neurones to form giant axons is known to occur. Syncytial connexions in neurone systems other than giant-fibre systems have also been described. A famous case is the enteric plexus of the leech (Apathy, cited and discussed by Bielschowsky, 1928); another is the atrial epithelial nervous system of *Amphioxus* (Boeke, 1935; Bone, 1958). We now have the first evidence for the simultaneous existence of a contact-system and a continuity-system in a coelenterate. In the open system of *Velella* the connexions are discontinuous and are of the type described as *en passant*. In the closed system the neurones form a syncytium. The obvious resemblance to the double nervous system in, say, a squid is enhanced by the large size of the fibres in the closed system. They are, in fact, giant fibres in the sense defined by Nicol (1948, p. 291).

Elsewhere in the Coelenterata continuity between neurones has sometimes been reported. Workers on *Hydra* agree that the ectodermal nervous system is of the continuous type (Schneider, 1890; Hadzi, 1909; McConnell, 1932; Semal-Van Gansen, 1952). In hydrozoan medusae Hertwig and Hertwig (1878) found that the subumbrellar plexus in *Carmarina* is continuous but that in other forms the junctions were of the contact type. In Scyphozoa and Anthozoa there have been conflicting opinions, but most recent accounts favour contact (Bozler, 1927; Woollard and Harpman, 1939; Pantin, 1952). Pantin's photos of the contact-system in *Metridium* make interesting comparison with my fig. 2 of the closed system in *Velella*. Although similar techniques were used in the two cases, the characters of the two nervous systems are obviously very different. It is not merely a question of different interpretations of a similar histological picture.

A completely satisfactory demonstration of continuity in the closed system
f *Velella* would require experimental investigation of the living tissue and
xamination by electron microscopy. This would be necessary because of the
emote possibility that the fibres in the system are composite structures, each
ormed by the interdigitation of minute terminal branches from separate
rocesses instead of by confluence of axoplasm as would be the case in a true
yncytium. Until such investigations are made, a slight element of doubt will
emain. One test of a syncytium—synchronized mitosis—cannot be applied
ere because the neurones appear to be post-mitotic, although provided with
entrioles.

unctional organization in double nervous systems

The behaviour of *Velella* is not sufficiently well known for an explanation
f the functions of the two nervous systems to be possible at this stage. In
he related form *Porpita* the behaviour is better known and there is a strong
kelihood that a double nervous system is present as in *Velella*. An investiga-
ion begun in 1954 on the nervous system of *Porpita* was held up for lack of
naterial. In my thesis (1956) I described continuous and discontinuous
unctions in the *Porpita* nervous system and further referred to 'association
ieurones' which are small neurones with fine processes, apparently associated
vith the main part of the nervous system in some specialized way. I now think
hat these association neurones probably correspond to the open system of
elella, although better preparations will be needed before this can be con-
rmed. The behaviour of *Porpita* (Mackie, 1959) includes local responses in
he organs of ingestion, co-ordinated tentacular movements, and curvature
f the mantle, the latter being a righting reaction, possibly of a geotropic
ature. The co-ordinated tentacular movements are of two sorts, one involv-
ng adoral flexions, the other aboral. Both are synchronized and, to the naked
ye, appear to be through-conducted. Should through-conduction prove to
ccur in *Velella* as well, the closed system discussed in this paper would im-
ediately come under suspicion as the probable conducting mechanism.

A system of giant fibres as well as a diffuse system occurs in scyphomedusae
Horridge, 1956*a*, *b*), the former conducting the contraction-wave during
wimming and the latter being concerned in a more generalized set of func-
ons. The giant fibres in these forms do not, however, form a continuum like
he closed system in *Velella*. In the hydrozoan medusa *Geryonia* there is
vidence of two conduction systems. Radial responses (between margin and
anubrium) can occur at the same time as circular responses (regular swim-
ing contractions) (Horridge, 1955). Two *histologically* distinct nervous
ystems have not been described in this form, however. It seems worth sug-
esting as a possible alternative to a double nervous system that in hydrozoan
edusae the subumbrellar nervous system is concerned with radial responses
nly, the impulses for swimming contractions being conducted by the muscle-
heet itself. One reason for suggesting this is that in the nectophores of several
phonophores that I have examined I have found no trace of a subumbrellar

nerve-plexus, although the two marginal nerve-rings characteristic of medusae are well developed. The implication is that in the absence of a manubrium and radial muscle, the subumbrellar nerve-plexus is not needed. There is also the small medusa *Eucopella* which lacks a manubrium and is said to be without a subumbrellar nerve-plexus (von Lendenfeld, 1883). It appears, too, that in certain Hydromedusae investigated (Krasińska, 1914, Taf. VII, 4), where radial muscle overlies circular, the nerves are associated with the radial muscle, lying superficially, and are thus separated from the circular muscle. The puzzling absence of nerves from the velum of Hydromedusae could be explained on similar grounds: that is to say that conduction across the entire striated muscle-sheet is myoid. There is therefore no definite evidence yet for a double nervous system (as opposed to two conduction systems) in any hydrozoan outside the Chondrophora.

Structural resemblances between nerve and muscle

Reference to myoid conduction raises the whole question of fundamental resemblances between nerve and muscle. It will only be possible to touch on one aspect of this question here, that of structural similarities.

Kleinenberg regarded the epithelio-muscular cells of *Hydra* as 'neuromuscular' cells in which the free end of the cell is sensory and the base muscular; sensory-cells, nervous-cells, and muscle-cells in more specialized tissues were viewed as arising in ontogeny or phylogeny from such precursors, by differentiation of the appropriate portions. Whether or not this conception is held to have any value, it is possible to see a family resemblance, as it were, between the anastomosing muscle sheets of *Hydra*, *Velella*, &c., and the nerve-nets also found in these forms. In both muscle- and nerve-nets the tissue originates from simple, separate cells which later send out processes in which fibrils develop. These processes grow together, forming the net in which the fibrils from adjacent cells associate intimately. The nerve-net can perhaps be likened to a muscle 'net' in which the cells are wider apart, where intermingling of fibres is concentrated at certain points, and where the fibrils are much smaller and have different physiological properties.

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The Fine Structure and Histochemistry of the Ligaments of *Nephtys*

By M. E. CLARK *and* R. B. CLARK

(*From the Department of Zoology, University of Bristol*)

With two plates (figs. 3 and 4)

Summary

A unique ligamentary system occurs in the polychaete *Nephtys*. Its fine structure and physico-chemical properties have been investigated.

The ligaments consist of alternating bands of elastic and inelastic elements, and they attach to the body by means of crystalloid attachment nodes. The nodes are probably glycoprotein in nature as they contain both protein and polysaccharide. They are not birefringent, and they are insoluble in comparison with the other components of the ligaments. Their structural stability is due mainly to strong ionic and hydrogen bonding. They resist enzymatic digestion.

The inelastic elements are bundles of thin, birefringent fibrils, 10 to 14 μ in length. The birefringence is positive with respect to the long axis of the fibrils and is primarily intrinsic in nature. The estimated coefficient of birefringence has a value between 10 and 20×10^{-3}. The fibrils consist of a protein with an isoelectric point near pH 6·3. The visible structure of the fibrils is due to both hydrogen and ionic bonding, while the birefringence is more dependent on hydrogen bonding. The fibrils resist heating and peptic digestion, but are slowly digested by trypsin. Of all the fibrous proteins, they most resemble collagen.

The elastic elements are extremely fine, granular cross-membranes bearing delicate filaments to which the ends of the fibrils are attached. They are continuous with a bounding membrane surrounding the ligament, which also bears granules and is also elastic. Some of the granules appear to be folds of the membrane itself. The membranes consist of a protein which is quickly digested by trypsin but not digested by pepsin. Its isoelectric point lies near pH 5. Hydrogen bonding is more important than ionic bonding in the membranes, and Van der Waals forces may also contribute. Classification of this protein is not yet possible.

Introduction

THE polychaete *Nephtys* possesses a system of ligaments which is, so far as is known, unique. The ligaments are conspicuous, shining, broad straps of tissue overlying the nerve-cord and running from there to the body-wall and parapodia, but neither their nature nor their function is clear.

They were discovered in *N. caeca* (Fabricius) by Ehlers (1864–8), who described something of the anatomy of the system. He observed the characteristic sheen of the bands and their woven or plaited appearance, but formed no opinion about their nature or function. Langerhans (1880) found similar ligaments in *N. hombergi* and *N. agilis* and observed that they were crossed by zigzag lines between which there occurred a fine, narrow, longitudinal striping. He concluded that the system of ligaments was an internal skeleton. *N. caeca* was re-examined by Schack (1886), but he added nothing to the two previous

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accounts. Emery (1887) made a detailed study of the anatomy of the ligamentary system and of the microscopic structure of the ligaments. He found that the woven appearance was due to alternating light and dark bands. He observed that the dark bands were birefringent and concluded that these structures were striated muscles and that the coelomic epithelium investing them was a delicate sarcolemma. In fact, it has been shown by Hoyle (1957) that they are not muscles.

We have observed these structures in the following species: *N. bucera* Ehlers, *N. caeca* (Fabricius), *N. caecoides* Hartman, *N. californiensis* Hartman, *N. cirrosa* Ehlers, *N. cornuta* Berkeley and Berkeley, *N. ferruginea* Hartman, *N. glabra* Hartman, *N. hombergi* Lamarck, *N. incisa* Malmgren, *N. longosetosa* Oersted, *N. magellanica* Hartman, *N. picta* Ehlers, *N. punctata* Hartman, *N. rickettsi* Hartman, *N. squamosa* Hartman; and we know of no member of the family Nephtyidae that lacks them. We have not made a detailed examination of the ligamentary system in all these species, but it appears that its anatomy and structure are the same throughout the family.

The structure of the ligaments has been examined by phase contrast and polarizing microscopy, and their chemical and histochemical properties have been investigated.

MATERIAL AND METHODS

Ligaments dissected from *N. cirrosa* and *N. hombergi* were used fresh, or fixed in 2·5% formalin, 10% formalin, or 35% alcohol as required.

The following histochemical methods were employed: biuret and ninhydrin tests for protein; Voisenet test for tryptophane; xanthoproteic reaction for phenols; nitroprusside test for sulphydryl and disulphide (all after Serra, 1946); lead acetate test for sulphur (Hawk, Oser, and Summerson, 1947); modified Thomas reaction for arginine (Liebman, 1951); periodic acid / Schiff (PAS) reaction after diastase treatment for polysaccharide other than glycogen; the standard Millon reaction for tyrosine; and saturated solution of Sudan black in 70% alcohol for lipid. For the arginine and PAS reactions, worms were fixed in Bouin and sectioned at 10 μ. Treatment with Sudan black was carried out on freshly dissected ligaments. In all other cases, dissected ligaments were fixed in 10% formalin and thoroughly rinsed in distilled water. In addition, the ninhydrin reaction was carried out on fresh ligaments as follows: (1) 0·2% ninhydrin in M/15 phosphate buffer, pH 6·98; (2) 0·1% ninhydrin in distilled water; (3) 0·1% ninhydrin in 2 M $CaCl_2$; (4) 0·1% ninhydrin in 2 M NaCl. The reaction in each case was carried out over boiling water for 1 to 2 min.

The reactions of fresh ligaments to the following reagents were also tested: 0·2 N HCl; 2 M $CaCl_2$; 6 M urea; saturated LiSCN; saturated $Ca(SCN)_2$; 98% formamide; 0·5 M, 1 M, 2 M, and 5 M NaCl; 0·25% trypsin (B.D.H.), pH 7·5 and 8·0; 0·25% pepsin, pH 4·58.

The effect of increased temperature on dissected ligaments was observed by mounting the specimen in distilled water or 20% ethyl alcohol on a slide.

coverslip was then sealed on with silicone grease. The slide was immersed a water bath which was warmed by a heated copper plate placed on the icroscope stage. Temperatures up to 85° C were obtainable. Changes in the ngth of small regions of the ligaments were followed with an ocular microieter.

The solubility of the ligaments at varying pH was studied. Freshly disected ligaments were immersed in buffers ranging from pH 1·45 to 12·00. or the pH range 1·45 to 9·20 a modified Michaelis acetate-barbiturate buffer earse, 1953) was used, with 0·196 M sodium barbital substituted for the odium barbiturate of the original. For the pH range 9·52 to 12·00, the örenson–Walbum glycine buffer (Pearse, 1953) was used. The pH of each olution was checked with an EEL direct-reading pH meter. In addition, the olubility of the ligaments was tested in 0·2 N HCl (pH 0·7). All these tests ere continued for 24 hours.

To determine the nature of the birefringence exhibited by these ligaments, ecimens were studied with the polarizing microscope after immersion for 4 h or more in the following media (refractive indices shown in brackets): ethylene iodide (1·756); 1-bromo-naphthalene (1·658); carbon disulphide ·627); aniline (1·586); terpineol (1·483); castor oil (1·477); chloroform ·466); 1-butanol (1·399); sea-water (1·34); distilled water (1·333). Measureents of retardation were made with a Berek compensator, in monochromatic ght from a sodium lamp.

RESULTS

icroscopic appearance of the ligaments

The ligaments are flattened ribbons lying in the coelomic cavity. There is paired, median ligament running in the midline on the dorsal surface of the entral nerve-cord. At two points in each segment it gives off several pairs of teral ligaments which run to the intersegmental body-wall and into the arapodia. The ligaments are attached both to the nerve-cord sheath and to he body-wall by means of small, hard 'attachment nodes'.

The intact ligaments vary considerably in width, not only among animals f different size, but also within the same individual. The larger ones partiularly have a silky, sometimes greenish, sheen in reflected light. In transitted light the ligaments are colourless or brownish yellow.

The ligaments have a very unusual microscopic appearance. They are comosed completely of extracellular, secreted material and are enclosed in a thin overing of coelomic epithelium. The attachment nodes have an irregular utline and a crystalline appearance, while the ligaments themselves are comosed of alternating dark and light bands, which may cross the long axis erpendicularly or diagonally. These dark and light bands, just visible with he dissecting microscope, give the appearance of striations, and when, as ften happens, the diagonal striping is irregular, a herring-bone or woven ppearance results (see fig. 1).

On closer examination, the ligament is seen to be made up of many fine, short fibrils which are grouped together side by side to form the dark bands. They connect at each end with an extremely fine, irregular, transverse membrane bearing small granulations, which occurs in the middle of the light bands. The fibrils are not attached to the membrane directly, but by means of a wavy filament which connects the fibril to a granule on the membrane.

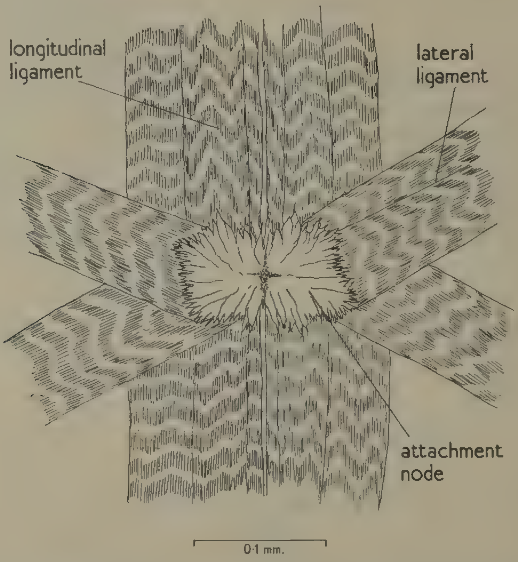
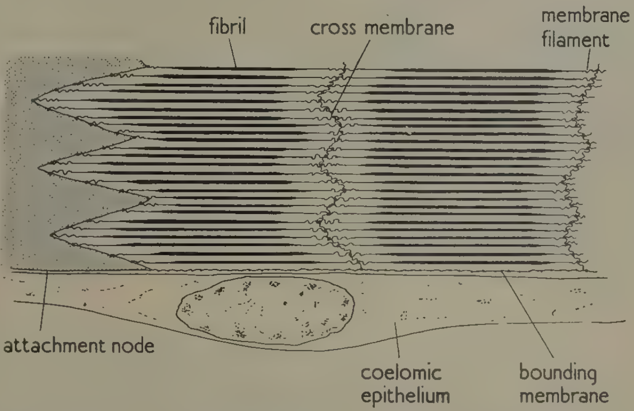


FIG. 1. A camera lucida drawing of the dorsal aspect of a ligament dissected from over the ventral nerve-cord, showing attachments to a node and the woven appearance of the ligaments.

Evidence of these has been found on observing the torn ends of ligaments where frequently the fibrils retain these filaments and the granules from the cross-membranes. The cross-membranes are continuous with a limiting membrane which surrounds the whole ligament. In larger ligaments there may be internal longitudinal membranes as well as the limiting one, so that these ligaments are actually composed of several large parallel units. Such longitudinal membranes can only be detected under special conditions, as described below. The fine structure of the ligaments, for which evidence is given later, is shown diagrammatically in fig. 2.

Ligaments treated with dilute trypsin or ligaments from sexually mature animals are quite fragile and partially disintegrate, releasing individual fibrils. These measure 10 to 14 μ in length (though occasionally fibrils are 20 or 21 μ long) and about 0·3 to 0·5 μ in diameter. The latter figure can only be estimated with the light microscope, as diffraction lines make exact measurement impossible. The range of length of the fibrils is the same in large

32 mm) and small (45 mm) worms. The longer fibrils are slightly thicker an the short ones. All appear to taper at their ends. Isolated fibrils are usually raight, but some are sharply bent near one end or, rarely, in the middle. his is probably an artifact resulting from damage during dissection. When gaments are torn, they invariably break cleanly at a cross-membrane, and hen they are bent in handling they bend most readily at these points.



IG. 2. A semi-diagrammatic representation of an enlarged portion of a ligament, showing the fine structure and relationships of the various components.

The ligaments are flexible and exhibit elastic properties. During dissection gaments were often stretched as much as twice their resting length before reaking. When the distortion was small, as it is during normal movements f the animal, an extended ligament retracted to its initial length as soon as e distorting force was removed. The ligaments are not always under tension the animal, however, since when the worm is contracted the longitudinal gaments fall into shallow folds.

When the ligaments were manipulated, it was found that stretching takes lace only in the light bands, but the complexity and small size of the structure involved made it impossible to detect exactly how this occurs. However, partially torn ligaments, isolated regions of the limiting membrane were een to stretch as much as two to three times before breaking, and this was llowed by instantaneous contraction. The limiting membranes, like the ross-membranes, bear granules and some of these disappear on stretching nd reappear on contraction, which may indicate that they are simply folds f the membrane. As will be shown below, no differences were found in the emical nature of the bounding membranes on the one hand, and of the ross-membranes and the wavy filaments by which the fibrils attach to them, n the other. It is reasonable to conclude that the cross-membranes and their wavy filaments are also elastic, though it cannot be said whether the elasticity of the isotropic regions is primarily due to stretching of the wavy filaments or to distortion of the cross-membranes with unfolding of some of the granules.

Birefringence of the ligaments

A most striking property of the ligaments is their high degree of birefringence. In polarized light they show alternating bright and dark bands, corresponding to the dark and light bands, respectively, seen in non-polarized light (figs. 3 A, B). The fibrils are responsible for the birefringence, and they can just be seen individually in the polarizing microscope. The birefringence is positive with respect to the long axis of the fibrils. In favourable preparations, the interband membrane also shows an extremely faint birefringence.

An attempt was made to determine the degree of intrinsic and form birefringence by immersing ligaments fixed in 2·5% formalin in media of various refractive indices, and measuring the retardation with a Berek compensator while illuminating with monochromatic light. Thickness was measured by focusing. The effect of formalin fixation on the birefringence was found to be nil by measuring the retardation of the same area of a ligament before and after fixation.

Because the packing of the fibrils varied considerably in different specimens and could not be accurately estimated, no exact values were obtained of the relative amounts of intrinsic and form birefringence. Isolated fibrils, because of their fine width, give far too small retardations for accurate measurement by this method.

However, certain conclusions are possible. On immersion in various media with refractive indices between 1·333 and 1·756, the birefringence never disappeared nor did it ever become faint. In methyl salicylate (R.I. = 1·537) the fibrils lost their refractility, and their birefringence was slightly decreased. Thus, the fibrils have a refractive index of about 1·50 to 1·55, and their birefringence is largely intrinsic.

While measurements of the coefficient of birefringence (retardation/thickness) obtained here are at best only rough estimates, the values fall between 10 and 20×10^{-3}, indicating a high degree of anisotropy for biological material.

According to Bear (1952), tannic acid treatment changes the sign of the intrinsic birefringence of collagen. This was tested on the ligaments, which

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FIG. 3 (plate). A, the torn end of a ligament seen in non-polarized light. The fibrils and irregular cross-membranes are just visible.

B, the same ligament as it appears in polarized light. Birefringence is associated with the fibrils.

C, part of a ligament viewed with phase contrast.

D, the same ligament immediately after treatment with buffer at pH 1·70. The fibrils have disappeared and the relationship of bounding and cross-membranes is apparent. A longitudinal membrane is also visible in the middle of the ligament. Granules can be seen on some of the membranes. Note lateral shrinkage of the ligament.

E, ligaments after treatment for 24 h at pH 3·90. The fibrils are not completely dissolved but have swollen and distended the membranes.

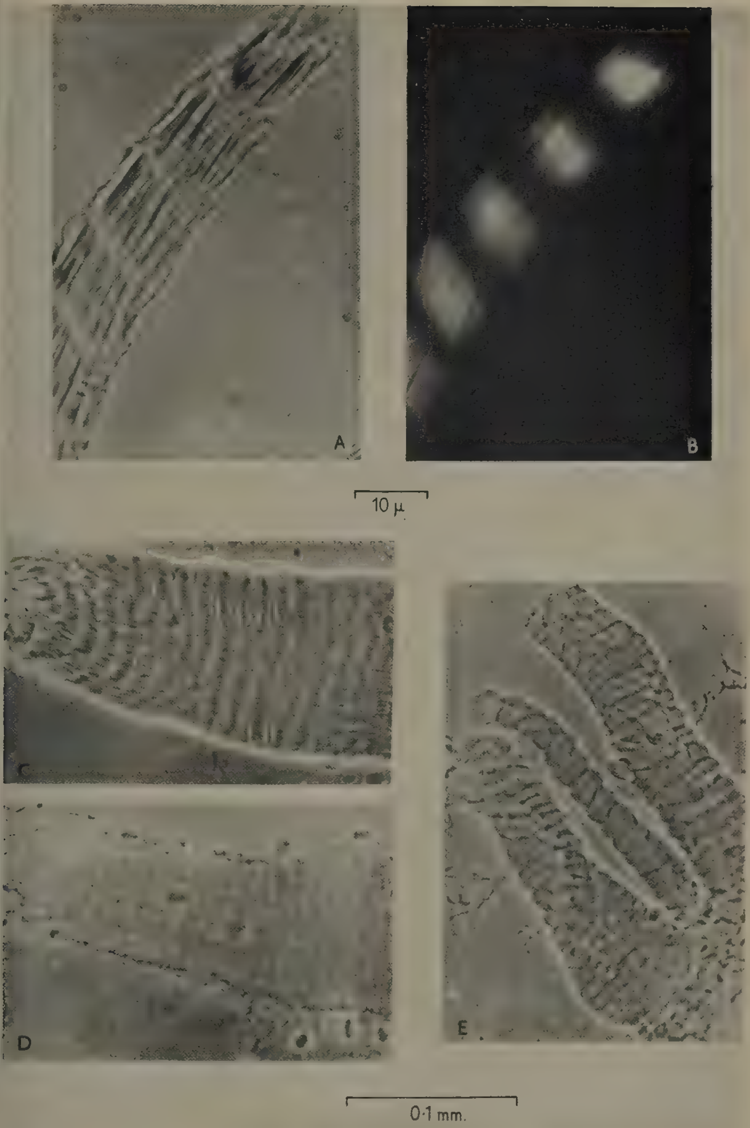

FIG. 3

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were treated for 3 days with 10% tannic acid, and then immersed for 24 h in methyl salicylate. At this refractive index, the contribution of form-birefringence to total birefringence would be near its minimum. Unlike that of collagen, the birefringence of the ligaments remained strongly positive.

Histochemical characters of the ligaments

The ligaments consist of 4 components: (1) the birefringent fibrils; (2) the interband and bounding membranes; (3) the attachment nodes; (4) the 'matrix' in which the fibrils lie. Of these, all but the last are microscopically distinct. The interband and bounding membranes are very thin, and it is difficult to judge their reactions in most cases. This applies as well to the delicate coelomic epithelium surrounding the ligaments, the reactions of which will also be considered.

The results are summarized in table 1. The biuret and ninhydrin tests show that the fibrils, membranes, and attachment nodes all contain protein. The attachment nodes react only moderately with the biuret test but give a strong blue colour with 0·1% ninhydrin in distilled water. On the other hand, the fibrils react readily with the biuret test but require treatment with 2 M $CaCl_2$ or NaCl before giving a moderate ninhydrin reaction. This suggests that ionic bonding of carbonyl and amide groups occurs in the fibrils, though not in the attachment nodes, and this prevents a reaction with ninhydrin. The interband and bounding membranes as well as the coelomic epithelium are biuret-positive and the matrix appears to be faintly positive also. However, the colour reactions of the matrix remain inconclusive since it is crossed in the interband region by the tapering ends of fibrils and by filaments from, and possibly folds of, the interband membranes, all of which are at or below the resolution of the light microscope.

The strong positive birefringence of the fibrils seems to preclude the presence of significant amounts of lipid or nucleoprotein in them. Alcohol-ether extraction for 24 h has no observable effect on the ligaments, and treatment with Sudan black shows mild lipid granularity only in the coelomic epithelium. The presence of polysaccharide material can be demonstrated in the attachment nodes by using the PAS technique after diastase treatment. No reaction was observed elsewhere in the ligaments, though the coelomic epithelium is faintly PAS-positive.

The ligaments were then tested for the presence of various amino-acids. Both the alkaline lead acetate test for sulphur and the nitroprusside reaction for protein-bound —SH and S—S are negative, which indicates that no significant amounts of amino acids containing sulphur are present. Both tyrosine and arginine are present in the fibrils and the attachment nodes. The latter, presumably because of their denser nature, give a more intense reaction in each case. The fibrils give a negative reaction for tryptophane, though the attachment nodes are strongly positive. This further confirms that they contain different proteins, which was already suggested by their differing reaction to the biuret and ninhydrin tests. Both fibrils and attachment nodes give a

TABLE 1

Reaction of various components of the ligaments to histochemical tests

	Biuret	*Ninhydrin*			*Xanthoproteic (tyrosine; tryptophane; phenylalanine?)*	*Voisenet (tryptophane)*	*Millon's (tyrosine)*	*Arginine*	*Alkaline lead acetate (sulphur)*	*Nitroprusside (SH and S—S)*	*PAS (polysaccharide)*	*Sudan black (lipid)*
		M/15 PO_4 *buffer*	*Distilled* H_2O	2 M NaCl *or* $CaCl_2$								
Attachment nodes	+	−	++	++	++	++	+++	+++	−	−	++	−
Fibrils	++	−	+(f)	++	++	−	++	++	−	−	−	−
Membranes	+	−	−	+(?)	+	−(?)	?	?	−	−	−	−
Matrix	+	−	−	−(?)	+(f)	+(f)	+(f)	+(?)	−	−	−	−
Coelomic epithelium	+(f)	−	−	+(?)	+(f)	+(?)	+(?)	+(f)	−	−	+(f)	+

+ = positive reaction; − = negative reaction; f = faint reaction; ? = uncertain result.

TABLE 2

Effect of various hydrogen ion concentrations on components of the ligaments

	pH 0·7	1·45	1·70	2·16	3·39	3·90	4·27	8·71	8·95	9·20	9·52	12·00
Fibrils												
Initial effect	d (fast)	d	d	d	s+d (slow)	s	o	o	o	o	d (slow)	d (fast)
After 24 h						no change	o	o	o	d		
Membranes												
Initial effect	d (fast)	o	o	o	o	o	o	o	o	o	d (fast)	d (fast)
After 24 h		o	o	o	o	o	o	o	d	d		
Attachment nodes												
Initial effect	d	o	o	o	o	o	o	o	o	o	o	d (slow)
After 24 h		o	o	o	o	o	o	o	o	d	d	

d = dissolves; s = swells; o = no observable effect.

positive xanthoproteic reaction of similar intensity. In view of the stronger tyrosine and tryptophane reactions in the nodes, this may possibly indicate a fair amount of phenylalanine in the fibrils, though no definite conclusion on this point can be drawn. (Hawk, Oser, and Summerson (1947) state that phenylalanine does not react under the usual conditions of the test, while Serra (1946) says that it does.)

Solubility at various hydrogen ion concentrations

The results of immersing freshly dissected ligaments in buffers ranging from pH 1·45 to 12·00 are given in table 2. The fibrils are insoluble over the pH range 4–9. The membranes have a wider range from 1·4 to 8·7, and the attachment nodes, which are the least soluble of the 3 in dilute acid and alkali, are insoluble between pH 1·4 and 9. As solubility is least at the pH of the isoelectric point (Cohn and Edsall, 1943) and increases nearly symmetrically on each side, the middle of the pH range of insolubility may be taken as an estimate of the isoelectric point. That of the fibrils is near pH 6·3, while the membranes and attachment nodes are more acidic, having isoelectric points near pH 5.

Solution of the fibrils in dilute acid is accompanied by an initial lateral shrinkage followed by a slow lateral swelling. The latter is best seen after treatment for 24 h in the pH range 1·45 to 3·90, when the membranes remain intact. As swelling occurs, the membranes are stretched, and the ligament increases in width. At pH 3·90, the fibrils do not dissolve, but there is some swelling (fig. 3, E). This is accompanied by an almost complete loss of birefringence.

The differential solubility of the components of the ligaments allows their chemical dissection, giving a clearer picture of how they are constructed. Fig. 3, C, D shows a freshly dissected ligament in distilled water and the same ligament immediately after treatment with buffer pH 1·70. It can be seen that the fibrils have disappeared and the membrane system has become more apparent in D. This membrane system is composed of a continuous sheath surrounding the ligament, and of cross-membranes corresponding to the isotropic, interband regions of the untreated ligament. Fig. 3, E shows a ligament after treatment for 24 h in buffer pH 3·90.

Results of enzymic digestion

Pepsin at a concentration of 0·25% and at pH 4·58 has no effect on any component of the ligaments even after 24 h. On the other hand, 0·25% trypsin at pH 7·50 readily digests the coelomic epithelium and the entire membrane system, freeing the fibrils. The latter are digested only slowly, digestion and the loss of birefringence being nearly complete after 24 h. The attachment nodes remain intact.

Tryptic digestion, therefore, affords a method of isolating the fibrils from the other components of the ligament. Fig. 4, A, B shows the same ligament before and after digestion with 0·25% trypsin at pH 7·50 for 15 min. Loss of the membranes and partial digestion of a cell of the coelomic epithelium can be seen. Fig. 4, C shows the fibrils dispersed by pressure on the coverslip, and fig. 4, D shows rows of fibrils, without their membranes but still more or less in position, and it also shows them separated from the attachment nodes. This suggests that the fibrils are attached to the nodes by filaments or membranes similar to those elsewhere in the ligaments.

Results of heat treatment

The distance from one interband membrane to the next was measured with an ocular micrometer on a ligament mounted in a water bath on the stage of the microscope. There is no change in length during heating of the ligaments from room temperature to 80° C, or after maintaining them at the latter temperature for as long as 1½ h. Ligaments heated while immersed in 20% alcohol also remain unchanged in length. There is no swelling in either water or alcohol.

After heating over a boiling-water bath 1¼ h, the fibrils and attachment nodes remain unchanged, but the interband membranes partially dissolve. After only 40 min there is a marked decrease in the birefringence of the fibrils, which suggests a disruption at the submicroscopic level. This means that very little of the fibrillar birefringence can be due to their microscopically visible anisometry.

Effect of lyotropic agents

The various bonds stabilizing structural proteins may result from Van der Waals forces, from tanning agents, and from electrovalent and covalent linkages (Brown, 1950). An attempt has been made to determine the degree and type of interpeptide chain bonding in the various proteins of the ligaments, especially the fibrils.

The high resistance to thermal agitation suggests that Van der Waals forces are not primarily responsible for the stability of the fibrils and attachment nodes, though they may contribute to the birefringent properties of the fibrils and to the structure of the interband membranes. The ready solubility of the ligaments in 0·2 N HCl rules out tanning agents, and indeed, fixation in formaldehyde confers resistance to acid treatment. The absence of positive results to sulphur tests and the solubility of the ligaments at moderate pH preclude any significant contribution from disulphide bonding. There remain hydrogen and ionic bonds to account for the structural stability of the fibrils and attachment nodes. Ionic bonds are broken by dilute acid and alkali and

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FIG. 4 (plate). A, part of a ligament viewed with phase contrast. Granules can be seen on the cross-membranes. The nucleus of a cell of the coelomic epithelium is lying against the ligament.

B, the same ligament immediately after treatment with 0·25% trypsin at pH 7·50. The cell of the coelomic epithelium and the membranes are partially digested, but the fibrils remain intact.

C, a region from the same preparation as B after pressure on the cover slip has dispersed the fibrils.

D, another region of C, showing separation of the fibrils from an attachment node (upper left).

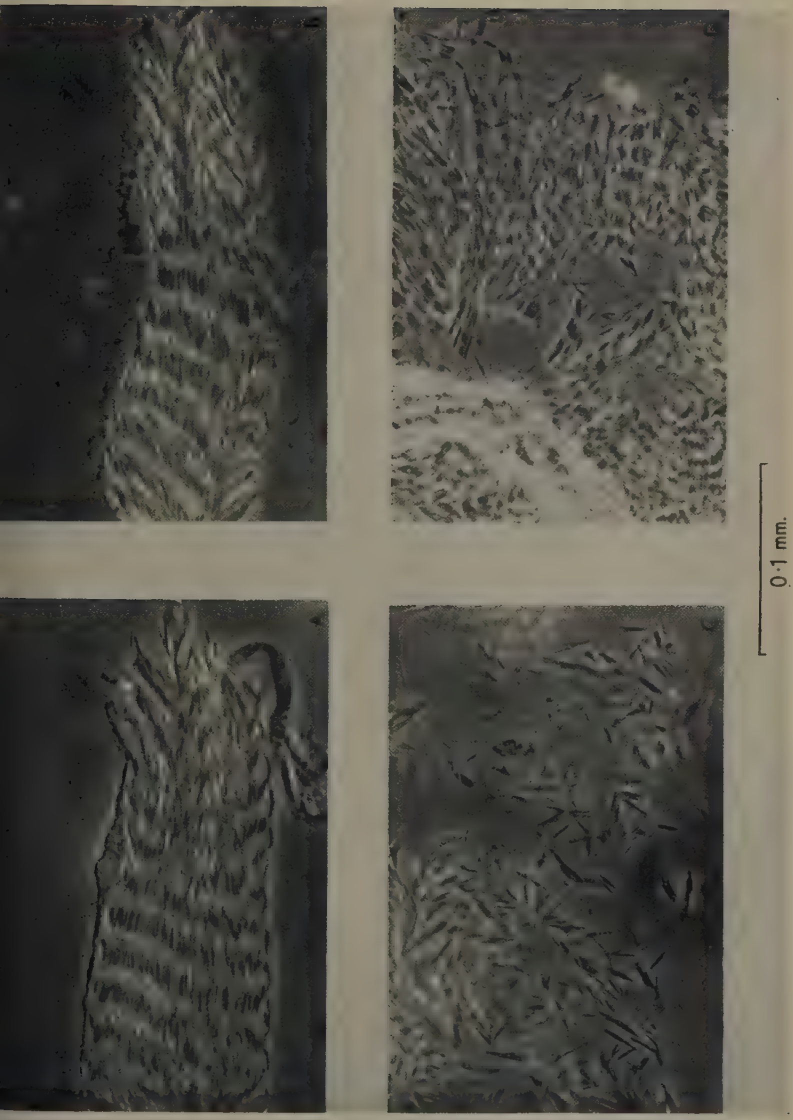

Fig. 4

M. E. CLARK *and* R. B. CLARK

by solutions of strong electrolytes, whereas both ionic and hydrogen bonds are broken by lyotropic agents (Bear, 1952). Whether the action of a given salt in dissolving a protein is due to its effect on ionic or hydrogen bonds can only be determined by comparison with the actions of other salts which are more or less lyotropic (Gustavson, 1949). NaCl, which has little lyotropic activity, serves as a base-line for the action of more lyotropic salts, such as $CaCl_2$, $Ca(SCN)_2$, and LiSCN. Proteins attacked by the latter but unaffected by NaCl must depend for their structural stability primarily on hydrogen bonds. For this reason, the effect on the ligaments of NaCl at various concentrations as well as of more lyotropic agents has been tested.

The results are shown in table 3. At no concentration does NaCl affect the membranes. It has its maximum effect on the fibrils at 1 to 2 M and on the attachment nodes at 5 M. In no case does it cause solution, but the effect on the fibrils is striking and unmistakable. During swelling they lose much of their resolution when viewed in ordinary light, though not in polarized light. On the other hand, lyotropic agents of similar ionic strength dissolve both fibrils and membranes. The birefringence of the fibrils is more susceptible to lyotropic action than is their visible structure. The attachment nodes are most resistant, and only dissolve readily in 98% formamide.

DISCUSSION

Of the four components of the ligamentary system of *Nephtys*, only the fibrils, attachment nodes, and membranes have been sufficiently characterized to warrant further discussion. Whether the matrix surrounding the fibrils is a dilute protein solution or is protein-free is not clear.

All three components are composed primarily of protein, though the attachment nodes also contain polysaccharide. The protein constituent of the attachment nodes differs from the proteins of both the fibrils and membranes not only in its resistance to solvents and to tryptic digestion, which may only be due to inaccessibility of its molecules through closer packing or to the presence of the polysaccharide, but also to its possession of a considerable amount of tryptophane, which is absent from the other two structures. The toughness of the nodes noted in handling, their crystalloidal appearance, and their relative insolubility suggest a close packing of the molecules, resulting from strong ionic and hydrogen bonds which are broken only by fairly strong acids and highly active lyotropic agents. The absence of detectable amounts of sulphur precludes any significant disulphide bonding. The absence of birefringence in the nodes probably means that the molecular packing is randomly oriented or that the micelles are nearly isometric. No conclusions have been drawn regarding the classification of this secreted, non-fibrous protein, though it seems possible that it is a glycoprotein.

The protein of the membranes differs from that of the fibrils in several respects, although the membranes are too small to show any differences with the usual histochemical tests. They are more readily susceptible to tryptic digestion. They also are stretchable and have been observed to possess elastic

TABLE 3

The effect of lyotropic agents on the ligaments

	Membranes		*Birefringence of fibrils*		*Visible structure of fibrils*		*Attachment nodes*	
	Initial effect	*After 24 h*	*Initial effect*	*After 24 h*	*Initial effect*	*After 24 h*	*Initial effect*	*After 24 h*
0·5 M NaCl	—	—	—	—	—	—	—	—
1·0 M NaCl	—	—	small decrease	same	swelling	same	—	swelling?
2·0 M NaCl	—	—	—	slight decrease	swelling	same	—	slight swelling
5·0 M NaCl	—	—	—	—	slight swelling	same	slight swelling	same
2·0 M $CaCl_2$	—	partial solution	slow decrease	disappears	less distinct	same	—	—
6 M urea	slow solution	—	rapid decrease	—	slowly dissolve	—	—	—
Saturated $Ca(SCN)_2$	slow partial solution and shrinkage	incomplete solution	disappears	—	immediate solution	—	—	swelling and slow solution
Saturated LiSCN	immediate solution	—	disappears	—	immediate solution	—	—	—
98% formamide	rapid solution	—	disappears	—	immediate solution	—	rapid solution	—

'Immediate' means solution on contact; 'rapid' means solution in sec, up to 2 min; 'slow' means solution in min, up to 2 h.

properties at distorting forces which have no effect on the fibrils. Indeed, no evidence has been found that the fibrils are elastic or even irreversibly extensible. The membrane protein has a much lower isoelectric point than has that of the fibrils, which indicates the presence of more acidic residues. It is also insoluble over a much wider pH range than the fibrils are, but is affected similarly by lyotropic agents. The membranes are unaffected by alcohol, but are partially destroyed after prolonged boiling, which has no effect on the visible structure of the fibrils though it decreases their birefringence.

The solubility of a protein depends on two opposing forces: (1) the lattice energy of the solid protein and (2) the energy of attraction between the dissolved protein and the solvent (Cohn and Edsall, 1943; Edsall, 1947). Three types of bonds contributing to the lattice energy must be considered here: ionic, hydrogen, and Van der Waals. As mentioned above, dilute acids and alkalis and solutions of strong electrolytes attack ionic bonds (Bear, 1952). The ions interact with the dipolar protein, producing hydration due to a Donnan equilibrium effect (Hermans, 1949), which results in a decrease in the activity coefficient of the protein and, consequently, in solution (Cohn and Edsall, 1943). On the other hand, both hydrogen bonds and ionic bonds are broken by lyotropic agents. The lyotropic action of salts can be estimated from their position in the Hofmeister series (Hermans, 1949). Other lyotropic agents include urea, formamide, &c. (Gustavson, 1949; Brown, 1950; Bear, 1952). Their loss of free energy on dilution is less than that of the protein concerned and consequently water passes from salt solution to the protein (Hermans, 1949). Van der Waals bonding is due to attractions between non-polar residues and is disrupted by organic solvents, which, however, decrease solubility of electrovalent bonds by decreasing the dielectric constant of the medium (Cohn and Edsall, 1943).

In the case of the membranes, insolubility over a wide pH range and in solutions of NaCl of varying ionic strength indicates either a high lattice energy of the ionic bonds or the presence of many hydrogen and/or Van der Waals bonds. That some hydrogen bonding is present is shown by the partial solution of the membranes in 2 M $CaCl_2$ (ionic strength = 6) but not in 5 M NaCl (ionic strength = 5) and by their total solution in 6 M urea. If Van der Waals forces from non-polar residues play a significant part in the lateral bonding of the membrane proteins, as they do in vertebrate elastin (Bear, 1952), there must be sufficient polar residues, giving rise to electrovalent bonds, to prevent their solution in organic solvents. Whether the small granules on the membranes are the same or a different protein is not clear. It seems most probable that at least some of them are simply regions where the membrane is folded, and that the decreased granularity when the membranes are stretched is the result of an unfolding process.

It would be premature to attempt to relate this protein with other types of fibrous proteins. The membranes are too fine to give definite colour reactions to histochemical tests, and for this reason the standard tests for vertebrate elastin have not been tried. Even in vertebrate tissues, these may give equivocal results (Hall, 1957). The membranes are less resistant than vertebrate elastin to acid treatment and to lyotropic agents (Hall, 1957; Partridge, Davis, and Adair, 1957). Digestion by trypsin used to be considered characteristic of elastin (Hawk, Oser, and Summerson, 1947), but it has since been shown that recrystallized trypsin does not digest elastic fibres (Lansing, 1952) and that digestion by crude trypsin preparations is due to the presence of pancreatic elastase (Baló and Banga, 1950). The trypsin used in our experiments undoubtedly contained both enzymes, and it cannot be said which of the two digested the membranes. Aside from the claims of Bouillon and Vandermeerssche (1957) that mesogloeal fibres in medusae are composed of elastin, this protein has not been reported in invertebrates. On the other hand, secreted fibres with elastic properties are not unknown (e.g. the byssus threads of *Mytilus* (Brown, 1952)), and although collagen fibres are not usually very extensible (Bear, 1952), collagen-like fibres may be (Picken, 1940; Brown, 1952). There is even some evidence of elastin being produced during life from collagen in vertebrates (Hall, 1957), and elastin seems more and more to be a member of the collagen group (Burton and others, 1955; Astbury, 1957). However, if the elastic membranes of the ligaments in *Nephtys* resemble collagen, they must have a very different organization from the fibrils.

With regard to the protein of the fibrils, there are two factors to consider: the structure giving rise to the birefringence, and the visible structure. The presence of much arginine in the fibrils coupled with their slightly acidic isoelectric point means that they possess sufficient acidic amino-acids to balance the basic arginine, and therefore they probably have considerable ionic bonding. This conclusion is supported by their solution on fairly small shifts in pH (±2·5 units) from the isoelectric point, their swelling and loss of resolution in 1–2 M NaCl, and the necessity of treating them with NaCl or $CaCl_2$ to obtain a satisfactory ninhydrin reaction. That the birefringence is hardly affected by NaCl but decreases sharply after treatment with the more lyotropic $CaCl_2$ and urea suggests that the birefringence is more dependent on hydrogen bonding, while the visible structure is due to both types of bonds. Van der Waals forces may play some part in producing the anisotropy, since prolonged treatment in boiling water decreases the birefringence.

The properties of the fibrillar protein so far described resemble those of collagens more than those of other fibrous proteins. It differs from most collagens in not being soluble in boiling water and in being digested slowly by trypsin. However, ichthyocol, a collagen from teleost skin, is also digested by trypsin (Gustavson, 1949), and elasmobranch collagen, elastoidin, is not transformed to gelatin on heating (Picken, 1940; Bear, 1952). Its positive birefringence, like that of collagen, is characteristic of all fibrous proteins. However, the sign of the intrinsic birefringence is not reversed by tannic-acid fixation, as occurs with some collagens (Bear, 1952).

Collagen-like proteins are known to occur in many invertebrates, and they have been reported in all three major annelid classes. Bradbury (1957, 1958) has described collagenous connective-tissue fibres in the Hirudinea, and the cuticles of both oligochaetes (Lumbricidae) and polychaetes (*Aphrodite*) yield the X-ray diffraction pattern of collagens (Rudall, 1955). However, the protein of earthworm cuticle differs from vertebrate collagen in having low thermal stability and in not being striated in electron micrographs, and, despite its collagen-like, wide-angle X-ray diagram, Gustavson (1957) is doubtful whether it should be considered a member of the collagen family. Smith (1957) has reported the presence of circumferential fibres originating from the sheath of the ventral nerve-cord and running around the body-wall in *Nereis*, but these appear not to be collagenous. Thus, while a type of collagen may occur in polychaetes, the ligaments of *Nephtys* are the only connective-tissue elements that are known which contain a probable collagen not directly associated with the cuticle or the sub-epidermal basement membrane.

The ligaments of *Nephtys* are also unique in being composed of alternating elastic and inelastic elements, the membranes and the fibrils respectively. The functional significance of such a structural organization will be considered in a following paper.

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The Ligamentary System and the Segmental Musculature of *Nephtys*

By R. B. CLARK *and* M. E. CLARK

(*From the Department of Zoology, University of Bristol*)

With two plates (figs. 14 and 15)

SUMMARY

Nephtys lacks circular body-wall muscles. The chief antagonists of the longitudinal muscles are the dorso-ventral muscles of the intersegmental body-wall. The worm s restrained from widening when either set of muscles contracts by the combined influence of the ligaments, some of the extrinsic parapodial muscles, and possibly, to a limited extent, by the septal muscles. Although the septa are incomplete, they can and do form a barrier to the transmission of coelomic fluid from one segment to the next under certain conditions, particularly during eversion of the proboscis.

Swimming is by undulatory movements of the body but the distal part of the parapodia execute a power-stroke produced chiefly by the contraction of the acicular muscles. It is suspected that the extrinsic parapodial muscles, all of which are inserted n the proximal half of the parapodium, serve to anchor the parapodial wall at the insertion of the acicular muscles and help to provide a rigid point of insertion for them.

Burrowing is a cyclical process involving the violent eversion of the proboscis which makes a cavity in the sand. The worm is prevented from slipping backwards by the grip the widest segments have on the sides of the burrow. The proboscis is retracted and the worm crawls forward into the cavity it has made. The cycle is then repeated.

Nephtys possesses a unique system of elastic ligaments of unusual structure. The anatomy of the system is described. The function of the ligaments appears to be to restrain the body-wall and parapodia from unnecessary and disadvantageous dilatations during changes of body-shape, and to serve as shock-absorbers against the high, transient, fluid pressures in the coelom, which are thought to accompany the impact of the proboscis against the sand when the worm is burrowing. From what is known of its habits, *Nephtys* is likely to undertake more burrowing than most other polychaetes.

INTRODUCTION

THE ligaments of *Nephtys* have attracted the attention of several authors (Ehlers, 1864–8; Langerhans, 1880; Schack, 1886; Emery, 1887), but only wo have made suggestions about their possible function. Langerhans (1880) hought that the ligaments formed an internal skeleton, a view which has never eceived support. Emery (1887), thinking the ligaments were striated muscles, supposed from their anatomy that they supplemented the extrinsic parapodial, acicular, and chaetal muscles. Hoyle (1957) disposed of the notion hat these structures were muscles by demonstrating their lack of electrical activity, and we have recently shown them to be composed of short lengths of a species of collagen, linked by elastic elements (Clark and Clark, 1960). The function of the ligaments is therefore still unexplained, though the

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anatomy of the ligamentary system suggests that it is likely to have a restraining influence upon changes in the shape of the worm. If this is so, the ligamentary system must be considered in relation to the anatomy and behaviour of the musculature.

Emery's (1887) account of the anatomy of the ligamentary system is substantially correct, but the accounts of the musculature of *Nephtys* by various nineteenth-century anatomists (Ehlers, 1864–8; Rohde, 1885; Schack, 1886; Emery, 1887) are all incomplete. They provide insufficient information for a functional analysis of the musculature to be made, and so we have re-examined the anatomy of the segmental musculature in detail before attempting to consider the function of the ligaments. Two species, *N. cirrosa* Ehlers and *N. hombergi* Lamarck, have been used for most of the work, but dissections have also been made of *N. bucera* Ehlers, *N. californiensis* Hartman, and *N. caeca* (Fabricius). No interspecific variation in the anatomy of the musculature has been detected, and from our experiments on the first two species it appears that the behaviour of the musculature is also constant.

ANATOMY OF THE MUSCULATURE

The division of the muscles under the headings body-wall musculature, extrinsic and intrinsic parapodial musculature, &c., is intended as a functional classification, but in the last resort, some arbitrary distinctions have had to be made.

Body-wall musculature

The familiar organization of the body-wall of annelids into segmented and paired, dorsal and ventral longitudinal muscles, and a ring of circular muscles, is highly modified in *Nephtys*. The chief peculiarity, as Ehlers (1864–8), Schack (1886), and Emery (1887) noticed, is the absence of circular body-wall muscles.

Dorsal longitudinal muscles (1) (figs. 1–3). The anterior insertion of these muscles is in the prostomium and the top and sides of the first 5 segments. Parts of these muscles encircle the pharynx, accompanying the circum-oesophageal connectives, and are inserted in the lateral lips of the mouth. These muscles taper markedly towards the posterior end of the worm. They are inserted into the dorsal and dorso-lateral intersegmental region and end in front of the anal muscle, in the last segment. The majority of fibres in the muscle-blocks cross the intersegmental boundaries without insertions into the body-wall. This is particularly marked in the anterior 30 to 35 segments, where few fibres are inserted dorsally in the intersegmental region, and the dorsal surface of the worm appears smooth. In the more posterior part of the worm a greater number of fibres have dorsal intersegmental insertions and there the segmental boundaries are marked by transverse grooves corresponding to the muscle insertions. In all segments the chief insertions are dorso-lateral, at the top of the grooves between the parapodia. The muscle-fibres are attached to a connective-tissue system which subdivides the muscle-blocks into a series of rather indefinite longitudinal compartments. The paired muscles meet in the mid-line and form an almost uninterrupted muscle-block across the top of the segments, and are folded around the sides of the gut.

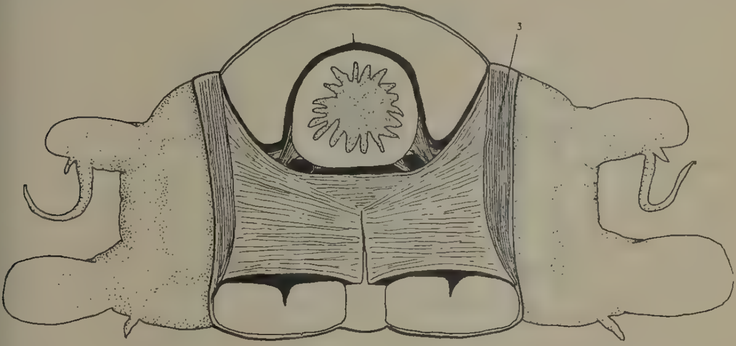

FIG. 1. Septal and proximal anterior dorso-ventral (3) muscles, viewed from the posterior side.

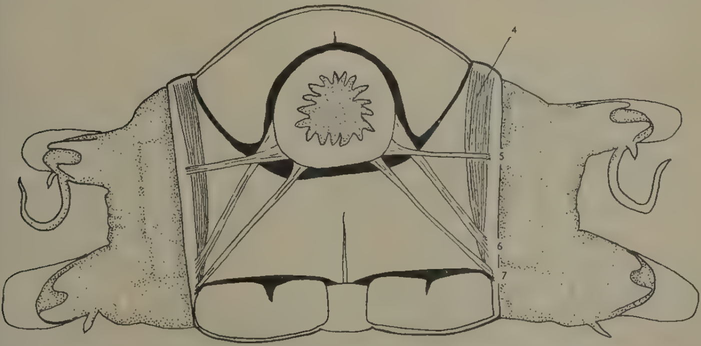

FIG. 2. Proximal posterior dorso-ventral (4) and intestinal (5-7) muscles, viewed from the anterior side.

Ventral longitudinal muscles (2) (figs. 1–3). The anterior insertion is in segments 1 to 5 along the sides of the folded membrane which joins the lateral lips of the mouth (see Clark, 1956*a*, fig. 1), and the posterior insertion is in the ventral part of the circum-anal, pygidial muscle-ring. There is a substantial insertion of muscle-fibres into each intersegmental groove on the ventral surface of the worm. The two muscles abut against the ventral nerve-cord medially and extend to the bases of the parapodia at the sides. Each muscle-block is folded into a U-shape.

Proximal dorso-ventral muscles (3, 4) (figs. 1, 2). Emery (1887) suggested that the dorso-ventral muscles and some of the chaetal muscles represented the circular body-wall muscles. This seems a reasonable interpretation, at least of the dorso-ventral muscles. They appear to be a continuation of the septal musculature on to the body-wall on each side of the intersegmental groove.

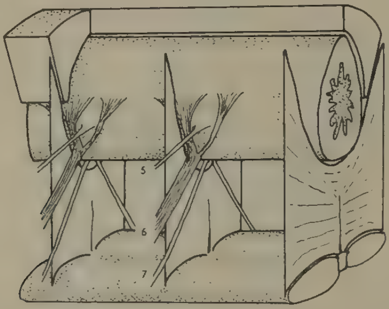

FIG. 3. Septal and intestinal (5–7) muscles. Antero-lateral view of two segments.

Septal musculature

The septum (figs. 1, 2, 3, 12) is attached mid-ventrally to the nerve-cord and laterally to the body-wall in the intersegmental groove. The lower edges of it cross the ventral longitudinal muscles and its upper edge passes beneath the gut and up the outer sides of the dorsal longitudinal muscle-blocks. The septum is not attached to either set of muscles, nor to the intestine. Thus the intersegmental septum is incomplete and the coelomic cavity of one segment is in communication with that of the next around the gut and between the septum and the ventral longitudinal muscles. There are no septa anterior to that between segments 34 and 35, because the anterior segments house the large, muscular, eversible pharynx.

The septum consists of a muscular sheet with an anterior and posterior covering of coelomic epithelium. There is no transverse connective-tissue membrane. The muscle-fibres in the dorsal part of the septum run transversely from body-wall to body-wall, but the majority of the fibres run in a fan from the body-wall to the ventral nerve-cord or to a mid-dorsal projection from it which runs two-thirds of the way up the middle of the septum. The free edges of the septum are thickened and its dorsal edge, beneath the gut, is folded over (fig. 12).

The gut suspension

In the anterior, non-septate segments, the gut is not attached to the body-wall, but is able to move freely and permit the eversion of the proboscis. In all the septate segments the gut is attached to the intersegmental part of the lateral body-wall. There is no medial dorsal mesentary, and no dorsal suspensory muscle to support the intestine; instead, the septa form a series of slings on which the gut rests. It is anchored down on them by 3 pairs of muscles in each segment immediately in front of the septum (figs. 2, 3).

Lateral intestinal muscles (5). These are inserted along the sides of the gut and run beneath the lateral edges of the dorsal longitudinal muscles to the posterior face of the parapodium, where they are inserted distal to the proximal dorso-ventral muscles (3 and 4).

Superior ventro-lateral intestinal muscles (6). These are inserted on the gut dorsal to the lateral suspensory muscles and their fibres become continuous with the circular muscle-coat of the intestine. They arise in two groups, a major one posterior to the lateral muscle and a very slight, minor one anterior to it. Both branches partly encircle the gut and join one another at the point where they diverge from the gut-wall and run to the parapodial wall. They are inserted distal to the dorso-ventral muscle (4), but many of the fibres in this intestinal muscle run into and become continuous with the dorso-ventral muscles. The superior ventro-lateral intestinal muscle is the best developed of the muscles inserted on the gut.

Inferior ventro-lateral intestinal muscle (7). This is inserted on the ventral surface of the gut on each side of the sub-intestinal blood-vessel, and runs to the body-wall at the base of the parapodium.

Extrinsic parapodial muscles

Six muscles arise from the mid-line, where they are inserted on the top and sides of the ventral nerve-cord, and run into each parapodium (figs. 4, 5). Of these the distal anterior and posterior muscles (8, 9) are the best developed. All these muscles are inserted in the neuropodium. The muscles inserted in the notopodium arise on the dorsal body-wall at the sides of the dorsal longitudinal muscles.

Distal anterior parapodial muscle (8). This muscle arises near the middle of the segment, where it is inserted on the top of the nerve-cord sheath, and runs to the anterior wall of the parapodium at the outer edge of the distal anterior dorso-ventral muscle (18) at the level of the top of the neuropodium.

Distal posterior parapodial muscle (9). This originates on the side of the nerve-cord sheath, beneath the anterior muscle (8). It runs to the posterior wall of the parapodium, where it is inserted between the proximal (4) and distal (19) dorso-ventral muscles.

Proximal anterior parapodial muscle (10). This originates on top of the ventral nerve-cord between the distal parapodial muscles (8, 9) and the septum, and runs to the anterior wall of the parapodium, where it is inserted at the inner edge of the dorso-ventral muscle (18).

Proximal posterior parapodial muscle (11). Originates immediately posterior to the distal anterior muscle (8), on the top of the nerve-cord sheath, and runs to the intersegmental groove.

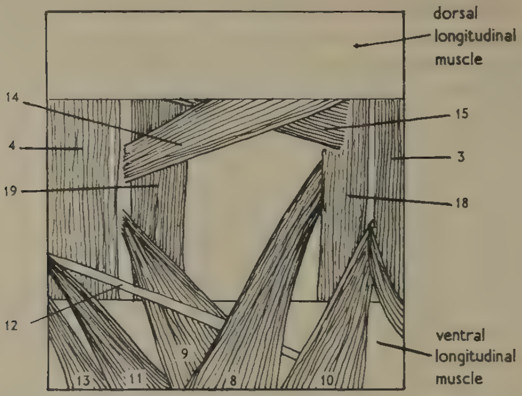

FIG. 4. Extrinsic parapodial musculature and the dorso-ventral muscles. Diagrammatic view of one segment viewed from the mid-sagittal plane towards the parapodium. The acicular muscles have been omitted. Anterior end to the right. 3, proximal anterior dorso-ventral muscle; 4, proximal posterior dorso-ventral muscle; 8, distal anterior parapodial muscle; 9, distal posterior parapodial muscle; 10, proximal anterior parapodial muscle; 11, proximal posterior parapodial muscle; 12, posterior diagonal muscle; 13, trans-septal muscle; 14, dorsal parapodial extensor; 15, dorsal parapodial flexor; 18, distal anterior dorso-ventral muscle; 19, distal posterior dorso-ventral muscle.

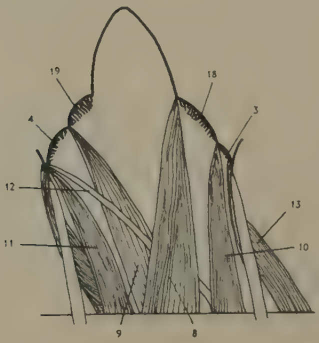

FIG. 5. Extrinsic parapodial musculature. Diagrammatic dorsal view. 3, proximal anterior dorso-ventral muscle; 4, proximal posterior dorso-ventral muscle; 8, distal anterior parapodial muscle; 9, distal posterior parapodial muscle; 10, proximal anterior parapodial muscle; 11, proximal posterior parapodial muscle; 12, posterior diagonal muscle; 13, trans-septal muscle; 18, distal anterior dorso-ventral muscle; 19, distal posterior dorso-ventral muscle.

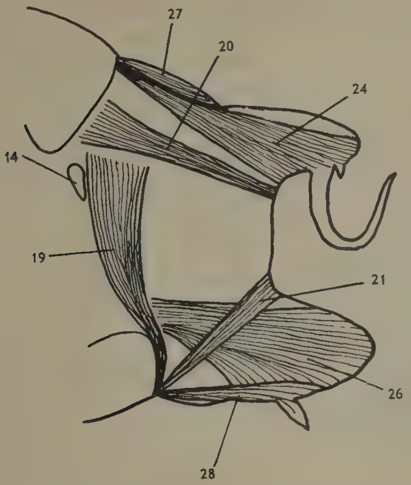

FIG. 6. Intrinsic parapodial musculature. Muscles on the posterior wall of the parapodium. 14, insertion of dorsal parapodial extensor; 19, distal posterior dorso-ventral muscle; other muscles not named.

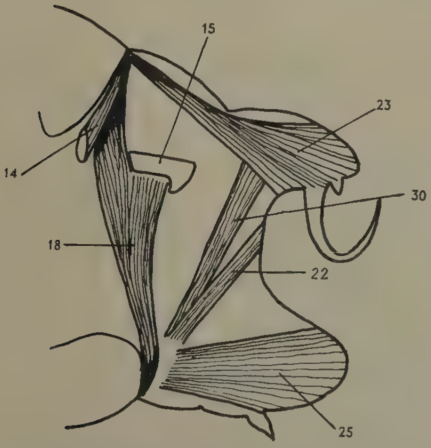

FIG. 7. Intrinsic parapodial musculature. Muscles on the anterior wall of the parapodium. 14, dorsal parapodial extensor; 15, insertion of dorsal parapodial flexor; 18, distal anterior dorso-ventral muscle; other muscles not named.

Posterior diagonal muscle (12). This originates at the side of the nerve-cord beneath the proximal anterior muscle (10) and runs diagonally beneath the distal anterior (8) and above the distal posterior (9) muscles to an insertion in the posterior wall of the parapodium near the intersegmental groove.

Trans-septal muscle (13). This arises in the extreme posterior part of the segment from the side of the nerve-cord sheath. It passes under the septum and is inserted on the anterior wall of the parapodium behind.

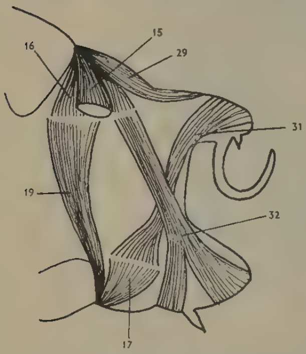

Fig. 8. Intrinsic parapodial musculature. Muscles in the posterior half of the parapodial cavity. There are similar muscles in the anterior half of the parapodium. 15, dorsal parapodial flexor; 19, distal posterior dorso-ventral muscle; other muscles not named.

Dorsal parapodial extensor muscle (14) (figs. 4, 6, 7). This originates in the dorsal part of the segment at the outer edge of the longitudinal muscle. Part of its insertion extends on to the anterior face of the parapodium. The muscle runs diagonally across the parapodium to an insertion on its posterior face between the proximal (4) and distal (19) dorso-ventral muscles, at the level of the lower edge of the dorsal longitudinal muscles.

Dorsal parapodial flexor muscle (15) (figs. 4, 7, 8). This muscle lies deeper in the parapodium than the dorsal extensor muscle (14). It originates in the dorsal posterior wall of the parapodium and crosses diagonally to a large insertion on the anterior face of the parapodium at the dorsal limit of the distal dorso-ventral muscle (18), and on its outer edge. It is considerably larger than the corresponding extensor muscle (14).

The intrinsic parapodial muscles

The intrinsic musculature of the parapodium is complicated and there is little to be gained by naming and describing all the muscles shown in figs. 6, 7, and 8, although the muscles have been numbered in the figures to simplify the following discussion of them.

Some of the muscles running to the tips of the parapodial lobes are inserted into the body-wall at the base of the parapodium, but the majority are inserted into 3 clearly defined regions on the anterior and posterior faces of the parapodium. These insertion areas are visible from the exterior as two horizontal grooves, near the upper and lower edges of the parapodium, and a vertical groove joining them (figs. 1, 2). All these regions of muscle insertion are in the proximal part of the parapodium. Muscles from the dorsal and ventral edges of the parapodium (16, 17) provide a support for the horizontal insertion regions. These supporting muscles are better developed on the posterior than the anterior wall of the parapodium because more muscles are inserted there.

The outer margins of the anterior (18) and posterior (19) distal dorso-ventral muscles are indicated by the vertical grooves in the parapodial walls. Both originate in the ventral body-wall beside the longitudinal muscle and run to the dorsal horizontal insertion area, though a slip of the anterior dorso-ventral muscle (18) continues medial to the large insertion of the flexor muscle (15) to an insertion in the dorsal body-wall.

Muscles run to the tip of the notopodium from the dorsal body-wall on both the anterior and posterior walls of the parapodium (23, 24) and there are corresponding muscles in the neuropodium (25, 26). Muscles also run from the body-wall to the dorsal and ventral parts of the parapodium (27, 28), but these do not extend into the tips of the notopodium or neuropodium. They are inserted into the posterior face of the parapodium and there is an additional dorsal muscle (29) in the middle of the parapodium. Two muscles on the posterior face of the parapodium are inserted in the upper and lower parts of the inter-ramal region (20, 21), and a third, on the anterior wall of the parapodium (22), runs to the middle of the inter-ramus.

The most conspicuous muscles lying in the cavity of the parapodium are a pair running from the proximal part of the neuropodium to the tip of the notopodium (30, 31), and a single muscle from the proximal part of the notopodium to the tip of the neuropodium (32). The former run against the anterior and posterior parapodial walls, while the latter runs between them in the cavity of the parapodium. It fans out and is inserted over the whole of the ventral part of the neuropodium.

The acicular muscles

There is one notopodial and one neuropodial aciculum in each parapodium. The bases of the acicula are close together and project slightly beyond the limits of the parapodium into the general body cavity. Each is provided with independent retractor and protractor muscles originating in the parapodial walls and inserted on a pad of connective tissue and aciculoblast cells at the base of the aciculum (fig. 9).

The retractor muscles are thin and delicate. One slender muscle (35) of the notopodial aciculum runs to the base of the intersegmental groove. The other notopodial (33, 34) and the neuropodial (36, 37) acicular retractor muscles radiate from their respective acicula to the parapodial walls between the proximal and distal dorso-ventral muscles.

The protractor muscles move the aciculum, and so the parapodium, backwards and forwards as well as laterally. They are inserted at the outer edge of the distal dorso-ventral muscles, though the dorsal anterior notopodial protractors (40) are inserted into the dorso-ventral muscle and its fibres become continuous with it. While all the protractor muscles are substantial, those originating in the anterior wall of the parapodium are better developed than the posterior muscles, and the anterior neuropodial protractors (43–45) are subdivided into 3 separate muscles originating at different levels in the parapodial wall.

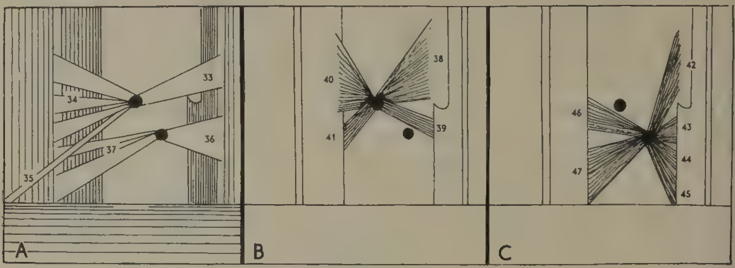

FIG. 9. Acicular musculature. Diagrammatic view of three segments viewed from the mid-sagittal plane towards the parapodium. The extrinsic parapodial muscles have been omitted. Anterior to the right. A, acicular retractor muscles; B, notopodial protractor muscles; C, neuropodial protractor muscles.

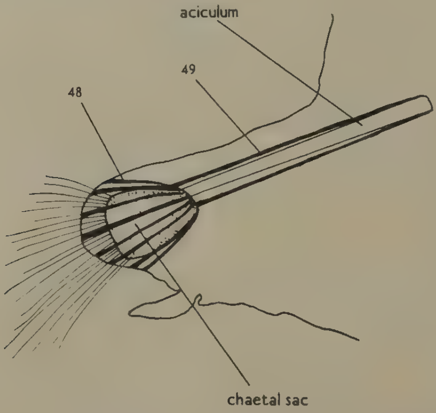

FIG. 10. Protractor (48) and retractor (49) muscles of the chaetal sac.

The chaetal musculature

The chaetal sacs lie in the tips of the noto- and neuropodia on either side of their respective acicula. The chaetal protractor muscles run in a cone from the sides of the chaetal sac to the parapodial wall (48). The retractors (49) run from the base of the chaetal sac to the base of the aciculum (fig. 10).

ANATOMY OF THE LIGAMENTARY SYSTEM

The anatomy of most of the ligamentary system was accurately described by Emery (1887), so that only a brief account of it need be given here.

The ligaments are flat straps of connective tissue, partly elastic, partly inelastic, that run along the dorsal surface of the nerve-cord to which they are attached at two points in each segment. From these points of attachment other ligaments run to the body-wall and into the parapodia (fig. 11). They are very conspicuous in the living worm and are glistening white with dark chevron-shaped or transverse striations on them, giving them a superficial resemblance to striated muscle, with which they were confused for many years. A detailed account of their structure has already been given (Clark and Clark, 1960).

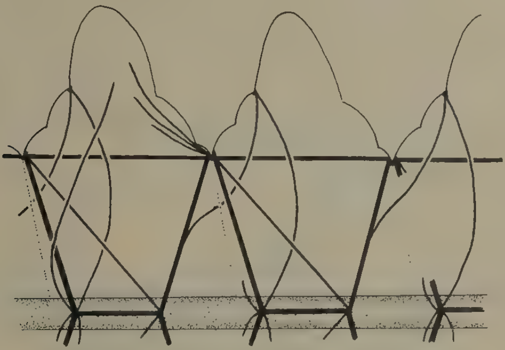

FIG. 11. Dorsal view of the ligamentary system (semi-diagrammatic). Anterior end to the right. In the left-hand segment all the ligaments are indicated; in the other segments only ligaments inserted on the body-wall are included. The ventral nerve-cord is stippled.

The ligaments are not attached directly to the nerve-cord sheath, but to a structureless, secreted attachment node which is, in turn, attached to the connective-tissue sheath investing the nerve-cord. The posterior attachment node is almost on the anterior face of the septum and is tilted upwards so as to be nearly vertical (fig. 12). The anterior insertion node is about three-quarters the length of the segment from the posterior node and is horizontal. A third important point of insertion of the ligaments is on the body-wall in the intersegmental groove at the level of the top of the neuropodium.

Three pairs of ligaments arise from the posterior attachment node on the nerve-cord. The most dorsal of these runs into the notopodium and is inserted into the posterior face of the acicular sac. The broader of the two ventral ligaments runs to the insertion on the body-wall in the intersegmental groove immediately posterior to it. The finer ligament runs into the neuropodium and is inserted in its posterior wall, where it is joined by a ligament from the anterior node. From this point ligaments run to the neuropodial aciculum and chaetal sac.

Two pairs of ligaments arise from the anterior attachment node on the nerve-cord. The more anterior of these is much the stouter and runs to the insertion on the intersegmental groove anterior to it, while a branch from it diverges, passes beneath the septum, and runs into the parapodium of the next anterior segment, where it is inserted on the posterior parapodial wall. The more posterior ligament arising from the node runs to the intersegmental insertion posterior to it, and is accompanied by the posterior diagonal muscle (12) (figs. 4, 5).

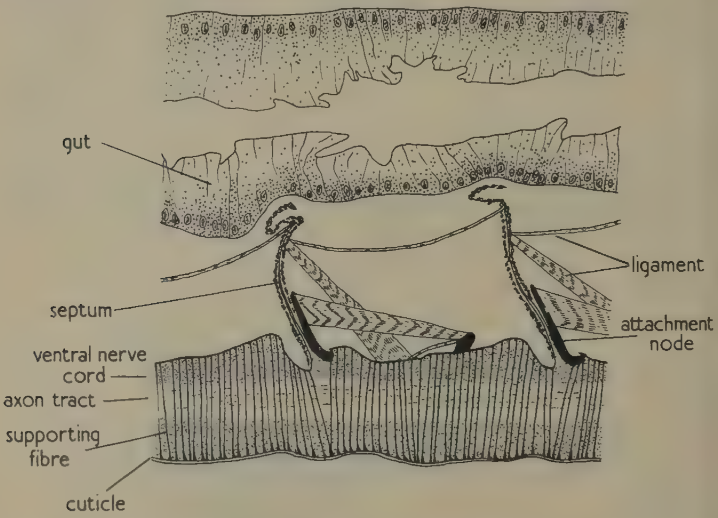

FIG. 12. Ligaments in the mid-sagittal plane. Anterior end to the right. The ligament attachment nodes on the ventral nerve-cord are shown in black.

Three ligaments arise from the insertion on the intersegmental groove of the body-wall and run into the parapodium of the segment behind. Two pass into the neuropodium, where they are inserted in the acicular sac and in the wall of the neuropodium near the ventral cirrus. The third ligament runs dorsally and is inserted in the wall of the notopodium near the chaetal sac. The attachment nodes on the body-wall are connected by a lateral longitudinal ligament on each side of the body. This runs over the ventral longitudinal muscle and is as broad as the longitudinal ligament on the nerve-cord.

A further series of ligaments in the mid-sagittal plane provides some support for the septum (fig. 12). The ligaments are inserted directly into the nerve-cord sheath in the middle of the segment, and run to the extension of the nerve-cord sheath in the middle of the septum. A fine ligament connects their point of attachment on the nerve-cord to the anterior attachment node. There is also a very fine ligament that runs from one septum to the next at a more dorsal level, near the free edge of the septum.

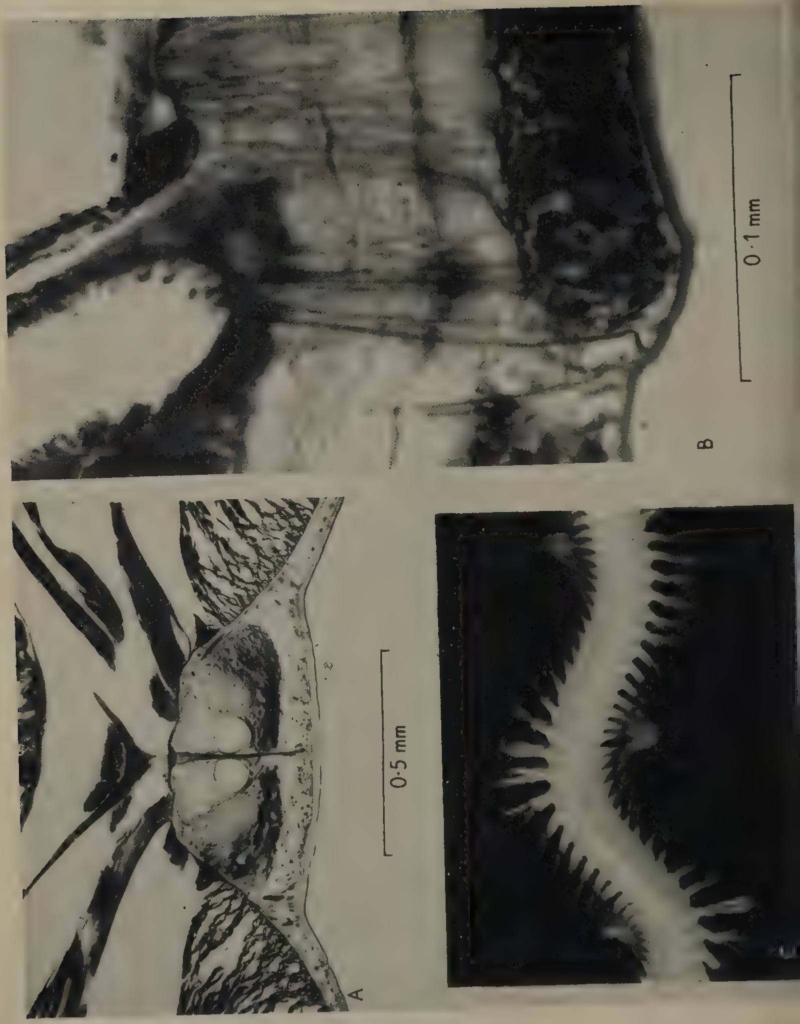

FIG. 14

R. B. CLARK *and* M. E. CLARK

MODIFICATIONS OF THE VENTRAL NERVE-CORD SHEATH

The inner (coelomic) side of the nerve-cord is bounded by a sheath which s an extension of the epidermal basement membrane. The ligaments, by way f their attachment nodes, the septa, and the extrinsic parapodial muscles are ll inserted on the sheath. The sheath is strengthened and supported in this egion by thickening and by a row of fibres forming a sort of palisade in the id-sagittal line connecting the nerve-cord sheath with the cuticle (figs. 12; 4, A, B). In transverse section it may appear as if the nerve-cord is bisected ngitudinally by a continuous membrane, but in fact there are spaces between djacent fibres. The modifications of the nerve-cord sheath for the attachment f muscles and ligaments is comparable to those in the ventral nerve-cord of ereids (Defretin, 1949; Smith, 1957).

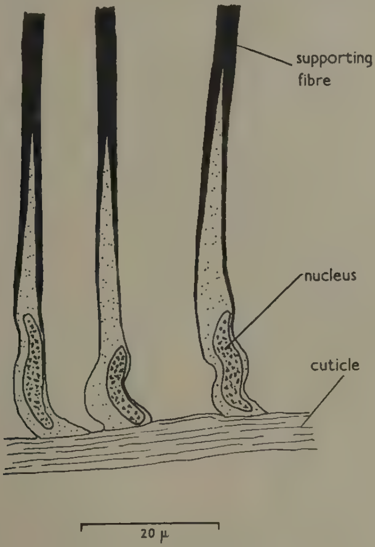

FIG. 13. Bases of the cells which secrete the supporting fibres of the ventral nerve-cord.

IG. 14 (plate). A, transverse section of the ventral nerve-cord posterior to the sub-oesophageal anglion, including a supporting fibre of the nerve-cord. Ligaments (dorsally) and extrinsic arapodial muscles (dorso-laterally) are inserted on the nerve-cord sheath. *Nephtys cirrosa*, icroformol, Holmes's silver technique.

B, mid-sagittal section of the ventral nerve-cord in the middle region of the body, including pporting fibres of the nerve-cord. Other structures may be identified by reference to fig. 12. *. hombergi*, Bouin, Heidenhain's 'Azan'.

C, enlargement of part of a swimming *Nephtys*. Anterior end to the right. Note the displacement of the posterior chaetae during the power-stroke of the parapodia.

Each fibre connecting the sheath to the cuticle is the product of a single cell (fig. 13). The cell-bodies are in contact with the cuticle and are 8 to 10 μ wide at the point of attachment, tapering to 3 to 4 μ wide 60 or 80 μ from the cuticle. The cell is slightly sinuous at its base and the nucleus, which is 20 μ long and 3 to 4 μ across, being situated in the basal part of the cell, follows its convolutions. The fibre is secreted at the cell boundary and runs as a solid homogeneous rod towards the nerve-cord sheath. The distal end of the fibre is frayed out and is inserted over an area perhaps 10 to 15 μ in diameter in the nerve-cord sheath. The fibres are highly birefringent and are straight and give the impression of being rather stiff and rigid. They are spaced at intervals of 10 to 20 μ and run more or less parallel to each other. The only wider gap than this between adjacent fibres is at the base of the septum, where the nerve-cord sheath is drawn up into a narrow cone running in the middle of the septum. Emery (1887) described this extension of the sheath as a nerve supplying the septal muscles. While we cannot be assured that there are no nervous elements in this structure, what Emery saw and described was certainly not a nerve, but an extension of the nerve-cord sheath, hollow at its base and containing neuroglial and matrix cells of the nerve-cord.

The sheath is thickened on the top and sides. The greatest thickening is on the dorsal surface of the sheath, where most of the muscles as well as the ligament attachment nodes are inserted. The mid-sagittal line of the sheath is particularly thick where the fibres connecting it with the cuticle are inserted. Minute filaments of sheath run into the muscle insertions and also into the attachment nodes of the ligaments.

BEHAVIOUR OF THE MUSCULATURE DURING CHANGES OF SHAPE

Most worm-like animals change their shape by the antagonistic contractions of circular and longitudinal muscles of the body-wall, acting about the coelomic fluid (or equivalent deformable but incompressible material) which serves as a hydrostatic skeleton (see e.g. Chapman, 1950, 1958). *Nephtys* lacks a circular muscle coat, although derivatives of the circular muscles may exist in the dorso-ventral muscles and possibly in some of the intrinsic parapodial muscles. In the absence of a complete ring of circular muscles, changes of shape must be accomplished by different means from those described in other worms.

Many active worms, such as earthworms, have intersegmental septa which are complete or nearly so. In *Lumbricus* the septa are entire, except for a ventral foramen which can be closed by a sphincter muscle, and they serve to divide the animal into a series of water-tight compartments (Newell, 1950). Under these conditions the changes of shape of one segment are almost independent of those of its neighbours. The septa of *Nephtys* are incomplete, but evidence is presented later to show that under certain conditions coelomic fluid does not pass from one segment to the next during muscular contractions. The behaviour of the septa cannot be observed directly, but the means by which they become effective barriers to the transmission of fluid pressures or of coelomic fluid from one segment to the next, can be inferred from their anatomy. The ventral, free edge of the septum is closely applied to the ventral longitudinal muscles (the space between the septum and the muscle has been exaggerated in figs. 1 and 2), and the outer edge of the septum is attached to the body-wall at the base of the inter-segmental groove, so that when the septal muscles contract the lower margin of the septum must be drawn tightly against the ventral muscle-blocks. Although it is not attached to the sides of the dorsal longitudinal muscles, there is no space between them. When the transverse septal muscles of the upper free edge of the septum contract, the intestine is raised and pressed tightly into the space between the two dorsal longitudinal muscles, and the cross-sectional shape of the gut is always such as to fill this gap completely. The only remaining space by which one segment is in communication with the next is at the lower lateral edge of the intestine, between it and the lower edges of the dorsal longitudinal muscle. From the anatomy of the intestine, the longitudinal muscles and the septum, it is not clear if it would remain open or not when the septal muscles were contracted. The gut may be slightly dilated by the pressure of the septum against it and fill this gap, or the gut suspensory muscles immediately anterior to the septum may block this space and prevent fluid passing from one segment to the next posterior one, though in this case they would clearly act as a one-way valve. It should be noted that a ligament supports the septum on its anterior face and therefore tends to press it against these muscles. It is also possible that a contraction of these muscles would draw the edges of the intestine out so that they came into contact with the longitudinal muscles.

A single segment, assumed to be hydrostatically isolated from its neighbours by the contracted septa, may be regarded as a rectangular box with muscles acting in the 6 bounding planes. Those in the horizontal plane are the dorsal and ventral longitudinal muscles, those in the vertical plane are the dorso-ventral muscles, and those in the transverse plane are the transverse septal muscles, the proximal parapodial (10, 11) and the trans-septal (13) muscles. Theoretically, contraction of the longitudinal muscles stretches the vertical and transverse muscles, and contraction of either or both of the latter restores he segment to its original length.

In fact, measurements from photographic enlargements of narcotized fully extended) worms, and of the same worms fixed in Bouin's fluid after ecovery from narcotization (contracted), show that there is little change in he width of the segments; changes in length are compensated by changes in height (table 1). This is to be expected. The transverse muscle-fibres of the septa are slight and appear too delicate to cause major changes in the shape of the segment. Further, on theoretical grounds, it is generally supposed that the septal muscles keep the septum taut during changes in the shape of the segment, but play no significant part in producing that change of shape Newell, 1950). The proximal and trans-septal muscles are more substantial and act in the transverse plane. They are so disposed as to be able to resist

TABLE 1

Average percentage change in the dimensions of the segments on contraction of the worm

	Worm A			*Worm B*			*Worm C*		
Segment	*Length*	*Width*	*Height*	*Length*	*Width*	*Height*	*Length*	*Width*	*Heigh*
10–20	−33·3	0·0		−43·75	0·0		−23·5	+6·25	
20–30	−17·6	0·0		−25·0	0·0		−20·0	0·0	
30–40	−20·0	−6·7	+20	−16·3	0·0	+10	−20·0	0·0	+11
40–50	−20·4	0·0	+30	−8·7	+4·5	+20	−20·8	0·0	+11
50–60	−17·9	0·0		−8·3	0·0	+10		0·0	+10
60–70	−15·3	0·0	+20	−25·6	0·0	+10	−9·5	0·0	+11
70–80	−10·3	−7·1	+30				−6·5	0·0	
80–90	−14·8	0·0	+11						
90–100	−20·8	0·0							
Average	−18·93	−1·53	+18·5	−21·28	+0·75	+12·5	−16·72	+0·89	+10·

(Figures for the change in height of segments are only approximate.)

lateral forces on the lower half of the segment walls, or to restore the shape of the segmental wall if it is deformed by contractions of muscles in other parts of the segment. There are no transverse muscles in the upper half of the segment, so that recovery from deformation of that part of the segment would be difficult. But this part of the segment is not deformed by contractions of the longitudinal and dorso-ventral muscles, presumably because its shape is determined by the underlying longitudinal muscles, and these are so massive as to be unaffected by changes in the internal fluid pressure except in a longitudinal direction.

Thus the dorso-ventral muscles are the chief antagonists of the longitudinal muscles, and contractions in the longitudinal plane of the segment are compensated by an increase in the height of the segment, and conversely. The septal muscles play, at most, a very minor role, and the proximal and transeptal muscles serve to restrict changes in the transverse dimensions of the segment. In this they are aided by the ligaments running from the insertions on the nerve-cord to the insertions on the intersegmental body-wall.

BEHAVIOUR OF THE MUSCULATURE DURING SWIMMING

Nephtys is a rapid and efficient swimmer and, when submerged, uses this method of progression instead of crawling over the substratum. The mechanism of swimming is essentially the same as that described in *Nereis* by Gray (1939). The body is thrown into sinusoidal waves which pass forwards along the body. In *Nereis* nearly all the segments are involved in this undulatory motion, and the prostomium traces out a similar path to that traced out by all the segments, but in *Nephtys* the long, muscular pharynx imparts a stiffness to segments 15 to 30, and they and the more anterior segments are not

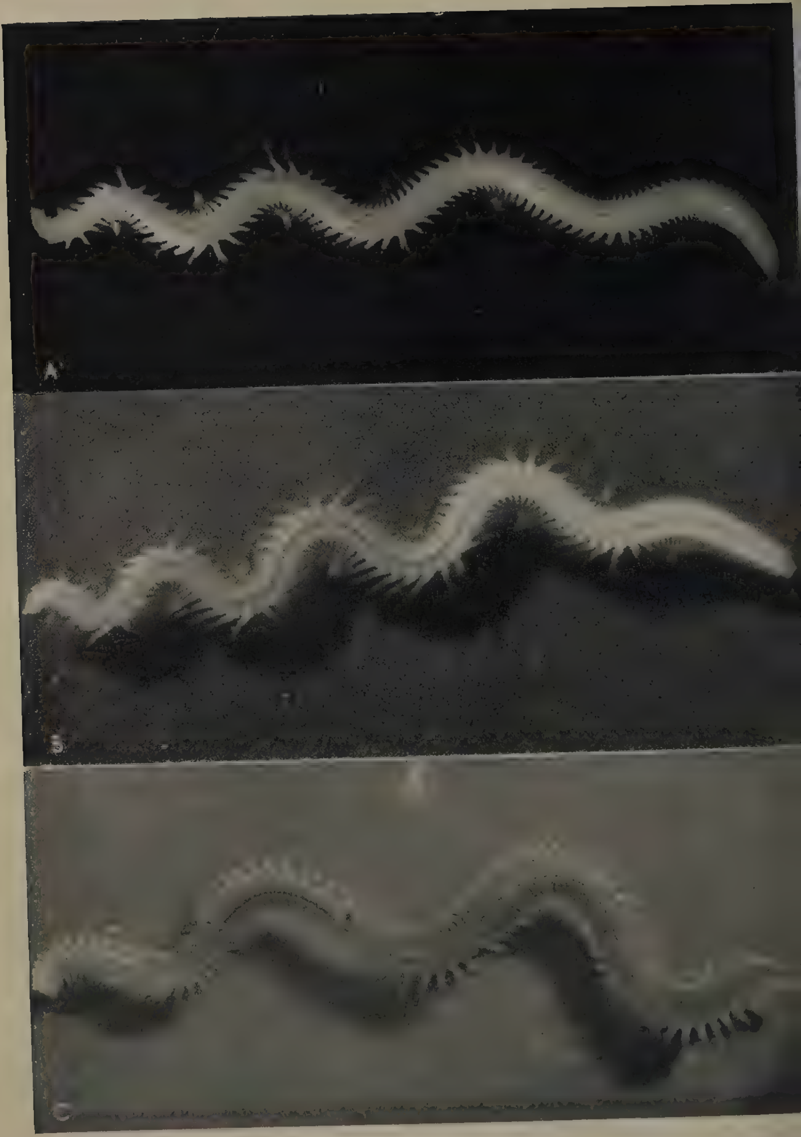

FIG. 15

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involved in the sinusoidal motion; instead, they execute a lateral pendulous movement (fig. 15, B).

Nearly all long, narrow animals swim in a similar manner. In smooth-bodied animals, such as eels, snakes, and leeches, the undulatory waves pass along the body in the opposite direction to the direction of motion of the animal, but in nereidiform polychaetes, the undulatory waves pass in the same direction as that of motion of the whole worm, i.e. from tail to head. Taylor (1952), in his theoretical analysis of the swimming of long and narrow animals, demonstrated that this reversal of the direction of motion of the locomotory waves relative to the direction of motion of the animal, could be related to the roughness of the body-wall. Roughness, such as that imparted by the presence of a series of rigid, lateral lamellae fixed at intervals along the body, is sufficient to cause the reversal of the locomotory waves, so that they pass from tail to head when the animal swims forward. This is the situation in the nereidiform polychaetes.

Gray (1939), in his earlier analysis of cinematographic records of swimming *Nereis*, had shown that the parapodia move backwards relative to the ground at the moment when the underlying longitudinal muscles are maximally elongated, i.e. when the crest of the undulatory wave reaches the parapodium. In addition to this passive role of the parapodia, resulting from the behaviour of the underlying longitudinal muscles during the passage of the locomotory wave along them, the parapodia also exert an additional back-thrust by executing a backwardly directed power-stroke at the moment when they are moving backwards relative to the substratum. The power-stroke executed by the parapodium is not essential for forward progression, as appears particularly from Taylor's analysis, and, indeed, Gray suggested that in *Nephtys* the parapodia were entirely passive. This, if true, would be puzzling, for *Nephtys* is a much better swimmer than *Nereis*, which makes very slow progress through the water. In fact, the power-stroke of the parapodia of the former is much more conspicuous than that in *Nereis*.

A direct comparison of the part played by the parapodia of the two worms can be made by considering the angular movement of the parapodium relative to the segment during the passage of a complete locomotory wave. Comparable conditions in the two worms can be assured by considering animals that are swimming at such a rate that the body is thrown into the same number of locomotory waves and by measuring the movement of the parapodia over a single locomotory wave of the same wavelength : amplitude ratio in each, as in figs. 15, A, C.

In *Nereis* (figs. 15, C; 16, 18) the parapodia are perpendicular to the surface of the segment when the underlying longitudinal muscles are contracted. As these muscles are relaxed, the parapodium is drawn back in a preparatory

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FIG. 15 (plate). A, *Nephtys* swimming slowly.

B, *Nephtys* swimming fast (wavelength of undulatory waves reduced, amplitude increased, greater relative movement of the parapodia).

C, *Nereis* swimming slowly.

stroke and then executes the power-stroke, which begins slowly about half-way up the leading edge of the locomotory wave, then increases in speed and continues until the parapodium is half-way down the trailing edge of the wave. A recovery stroke then restores the parapodium to the perpendicular position. An important fact to notice is that the power-stroke continues over about half the complete locomotory cycle of the segment.

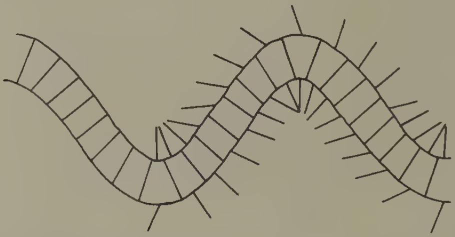


FIG. 16. Diagram showing the inclination of the parapodia of *Nereis pelagica* in relation to the position of the segments in the locomotory cycle. The undulations pass from left to right.

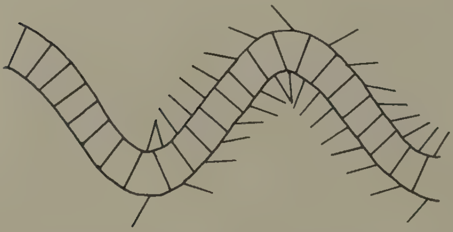


FIG. 17. Diagram showing the inclination of the parapodia of *Nephtys hombergi* in relation to the position of the segments in the locomotory cycle. The undulations pass from left to right.

In *Nephtys* (figs. 15, A; 17, 19), under comparable conditions, the resting position of the parapodium is inclined forward at an angle of 25° to 30°. About half-way up the leading edge of the locomotory wave, the parapodium is drawn further forwards in preparation for the power-stroke, which begins as the parapodium is carried up to the crest of the wave and is completed as soon as the parapodium has passed the crest on to the trailing edge. There is then a slow recovery to the resting position. In *Nephtys* the power-stroke is executed only while the parapodium is on the leading edge and the crest of the locomotory wave, and it occupies a smaller fraction of the complete cycle than it does in *Nereis*. This is indicated by the steeper slope of the power-stroke in fig. 19 than in fig. 18.

Faster progression through the water is achieved by increasing the number of locomotory waves into which the body is thrown (fig. 15, B), with the effect that the wavelength : amplitude ratio is decreased. Under these conditions in *Nephtys* the movement of the parapodium relative to the segment is modified (fig. 20). The resting phase of the parapodial cycle is almost abolished and the recovery stroke tends to merge into the preparatory stroke. Both the angle through which the parapodium is moved and its rate of movement are increased.

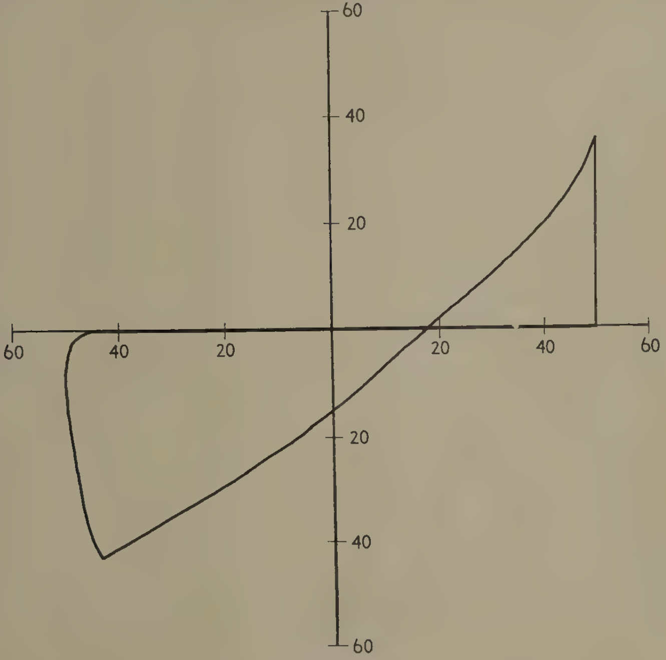


FIG. 18. *Nereis*. Diagram showing the change in inclination of the parapodium to the transverse axis of the segment during its passage through one complete locomotory cycle. Wavelength : amplitude ratio, 3·6. Horizontal axis: inclination of the longitudinal axis of the segment to the direction of motion of the worm. Vertical axis: inclination of the parapodium to the transverse axis of the segment. This diagram should be compared with fig. 15, C, from which it is derived. See text for further explanation.

Evidence that the movement of the parapodium relative to the segment is an active one and is not passively produced, for instance by local eddy currents produced by the locomotory waves, can be gained from fig. 14, C. The long and rather flexible posterior chaetae of the parapodium become swept in an anterior direction as the parapodium is moved backwards during the power-stroke, whereas the stiffer, anterior chaetae are not. There is thus a relative movement between the tip of the parapodium and the surrounding water which can only be produced by muscular activity.

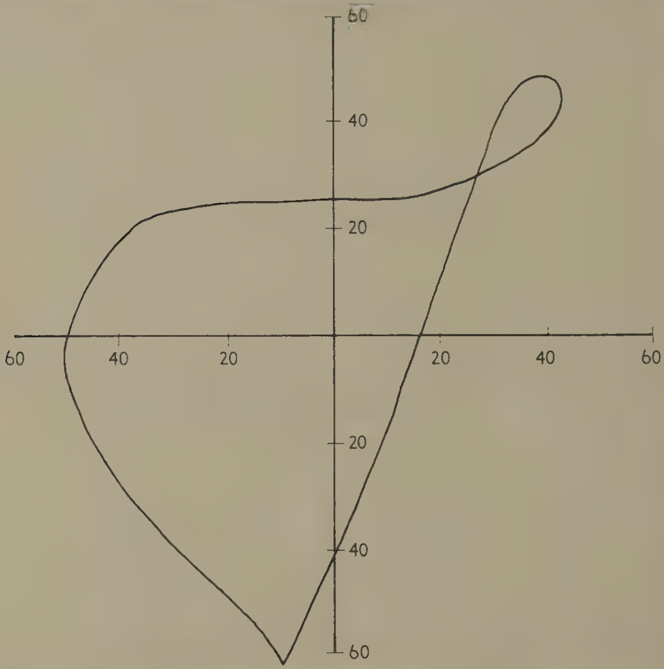
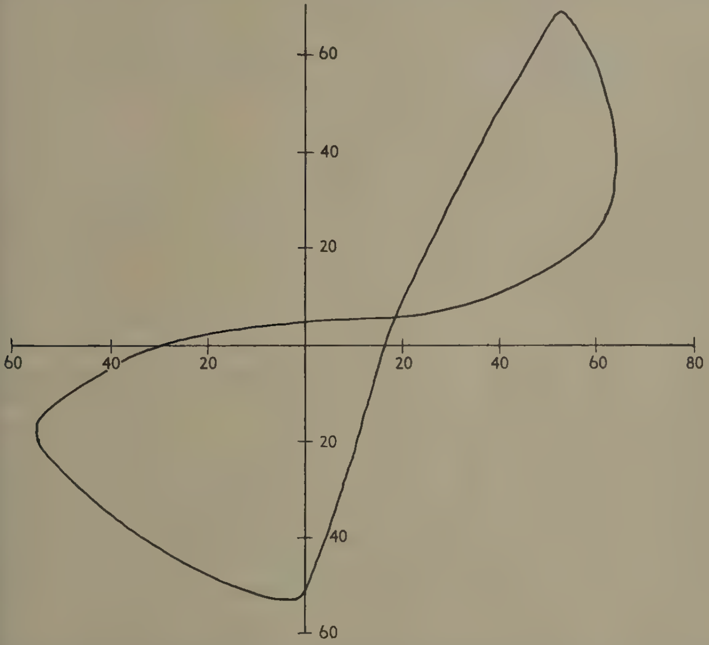


FIG. 19. *Nephtys*. Diagram showing the change in inclination of the parapodium to the transverse axis of the segment during its passage through one complete locomotory cycle. Wavelength : amplitude ratio, 3·5 (i.e. a comparable body form to that of *Nereis* in fig. 18). This diagram should be compared with fig. 15, A, from which it is derived. See fig. 18 and text for further explanation.

It will be noticed that the movement of the parapodium in *Nephtys* is confined to the distal half of the parapodium, while the basal part moves hardly at all in relation to the segment (figs. 14, C; 15, A, B). This can only be due to contractions of the acicular muscles, for none of the extrinsic parapodial muscles is inserted in the distal part of the parapodium (fig. 5). In any case, the extrinsic muscles are poorly disposed for producing powerful movements of the parapodium, for those inserted on its anterior face arise in the anterior part of the segment and those on its posterior face in the posterior part of the segment. Only 3 muscles run diagonally across the segment. One, the slender posterior diagonal muscle (12), is inserted in the intersegmental groove and can have no effect on the movement of the parapodium. The other two are he dorsal flexor and extensor muscles (14, 15), and these are inserted in the asal part of the parapodium. The chief propulsive force is therefore generated y the acicular muscles in conjunction with the longitudinal muscles.

This is not to say that the extrinsic parapodial muscles play no part in wimming. The contraction of the longitudinal muscles during the passage



IG. 20. *Nephtys.* Diagram showing the change in inclination of the parapodium to the ransverse axis of the segment during its passage through one complete locomotory cycle. Vavelength : amplitude ratio, 2·7 (i.e. rapid swimming). This diagram should be compared with fig. 15, B, from which it is derived. See fig. 18 and text for further explanation.

f locomotory waves reduces the volume of one-half of the segment and there s a compensating increase in the volume of the opposite half of it. Compared vith the segmental body-wall, the parapodial walls are thin and would be eformed by these pressure changes but for restraining influences. The basal art of the parapodium is restrained by the extrinsic muscles, and the distal art, which does become distended when the longitudinal muscles on the pposite side of the body are contracted, are prevented from too great a dilatation by the ligaments. Since the parapodia are inflated to only a very limited xtent, the reduction in volume of one half of the segment by the contraction f the longitudinal muscles is compensated by an increase in the length of the ongitudinal muscles on the opposite side of the body.

One of the chief problems in soft-bodied animals is the provision of rigid insertions for the muscles that move such skeletal elements as the animals possess. Without rigid insertions, much of the mechanical advantage gained by the use of skeletal rods is lost. In the case of the acicula of *Nephtys*, rigid insertions must be provided on the parapodial walls for the acicular muscles to produce the power-stroke of the parapodium. The power-stroke is performed while the underlying longitudinal muscles are being stretched and the parapodium is turgid. The acicular muscles are inserted in the parapodial walls near the insertions of the extrinsic muscles and these points appear to be held taut by the balanced and opposed forces of inflation by hydrostatic pressure and restraint by the extrinsic muscles.

While the same general principles obtain in *Nereis*, both the arrangement and the performance of the muscles differ from those in *Nephtys*. In *Nereis*, as in *Nephtys*, muscles arising from the ventral nerve-cord and the dorsal body-wall are inserted in the parapodial walls, but in the former worm some of those inserted in the anterior wall of the parapodium arise in the posterior part of the segment, and those on the posterior wall, in the anterior part of the segment (Snodgrass, 1938; Defretin, 1949), and so give a greater mechanical advantage. Further, the ventral muscles inserted in the anterior face of the parapodium run to the tip of the neuropodium (personal observation) and are chiefly responsible for producing the power-stroke. In *Nephtys*, the comparable muscles are inserted in the basal part of the parapodium and, as we have seen, do not serve the same function. In *Nereis* the power-stroke involves the whole parapodium, and it is probably effected chiefly by the extrinsic parapodial muscles. It is significant that in the heteronereis, which is a much faster swimmer than the immature worm (Lillie and Just, 1913, &c.), the muscles chiefly responsible for producing the power-stroke are much enlarged (Defretin, 1949; Durchon, 1952). The acicular muscles may play a subsidiary role and augment the effect of the extrinsic muscles, because those inserted on the anterior wall of the parapodium, and so capable of moving the parapodium backwards during a power-stroke, are larger than the posterior muscles.

Behaviour of the Musculature during Burrowing

Under suitable conditions, *Nephtys* buries itself in the sand very efficiently, and burrows rapidly through it. The initial penetration of the substratum and the progress through it are two distinct and clearly defined processes involving entirely different types of muscular activity.

Penetration of the substratum

When covered by water and provided with a substratum, *Nephtys* burrows into it by one of two methods. It may perform undulatory swimming movements while lying on the substratum. This does not result in any appreciable forward movement, but it agitates the sand and sweeps it on to the dorsal surface of the worm. Within a short time it is buried and then its behaviour changes and it begins to burrow through the substratum. Alternatively, it may swim through the water and approach the substratum obliquely. When the prostomium is in contact with the sand, the worm executes fast swimming movements which impart a rapid, low-amplitude, lateral movement to the prostomium and anterior segments. The agitation of the sand, combined with the forward thrust due to the swimming motion, results in the prostomium and the first 15 or so segments being buried within a short time. Swimming then ceases and burrowing begins. According to Chapman and Newell (1947), penetration into the substratum in this manner depends upon the exploitation of the thixotropic properties of the sand, which is rendered more easily penetrated by agitation.

It has been demonstrated in another context (Clark, 1956*b*) that contact between the dorsal surface of the worm and a solid body inhibits swimming activity and both these methods of gaining entry into the sand have in common the fact that they begin with swimming, which ceases when some part of the body is covered with sand, and the locomotory behaviour then changes to burrowing.

Burrowing through the substratum

Burrowing in the sand involves the use of the proboscis. Briefly, the proboscis is suddenly everted, punching a hole in the sand, into which the worm crawls when the proboscis has been retracted. Obviously, this method of burrowing depends upon the worm being anchored when the proboscis is everted; otherwise its impact against the sand would merely push the worm backwards instead of making a hole in the substratum. Accordingly, this type of locomotion is not observed unless at least 10 segments are buried and these provide the necessary anchor. Occasionally a worm placed on damp sand, but not under water, will attempt to penetrate into the sand by everting its proboscis. Unless the sand is extremely damp and quite soft, this is an unsuccessful manœuvre and the worm merely succeeds in raising its anterior segments from the surface of the sand. A similar phenomenon can also be observed when a worm attempts to burrow in coarse sand in an aquarium when the sand grains lack cohesion and the animal is unable to achieve the necessary anchorage.

The eversion of the proboscis is accomplished chiefly by fluid pressure generated by contraction of the longitudinal muscles, assisted, perhaps, by the contraction of the dorso-ventral muscles. The dorsal longitudinal muscles of the anterior 30 to 35 segments are particularly important. The limit of strong contraction of these muscles is indicated by a deep transverse furrow that appears across the dorsal surface in the region of the 30th to 35th segment, where they are inserted. It will be recalled that there are only slight intersegmental insertions of the dorsal longitudinal muscles in the more anterior segments. The first 34 segments which house the inverted proboscis are non-septate, with the result that increases in the fluid pressure are readily transmitted to the anterior end of the worm. The contraction of the dorsal longitudinal muscles draws the prostomium and the dorsal part of the first 5 segments upwards at a sharp angle to the rest of the body. Parts of these muscles are inserted at the sides of these segments and also run around to insertions in the lateral lips of the mouth. When they contract, the lips and the walls of the segments are drawn aside, so permitting the eversion of the large, muscular pharynx through these very much smaller segments. The folded membrane which joins the lateral lips is stretched tight beneath the everted proboscis. The modifications of the anterior nervous and blood-vascular systems in the first 5 segments, where the eversion of the proboscis entails considerable movement and distortion, have been described elsewhere (Clark, 1956*a*, 1958).

When the proboscis is everted, the force with which it is driven against the substratum is limited by the frictional forces between the worm and the substratum that prevent it slipping backwards. Only the widest segments, from about segment 15 to segments 45–50, are broad enough to reach the sides of the burrow that has been excavated by the proboscis in previous cycles of burrowing activity, and these form an anchor. The parapodia of all the segments are directed backwards when the proboscis is everted. Those of the most posterior segments are pressed tightly against the sides of the body; those of the middle, anchoring segments are held out at a slight angle. Segments 15 to 34 become reduced in volume by the contraction of the longitudinal and dorso-ventral muscles, which drives the coelomic fluid forwards into the everting proboscis, but they do not become appreciably narrower.

The passage of coelomic fluid across the segmental boundaries can be followed under favourable conditions by injecting a strong methylene-blue solution in sea-water into some of the segments. It can then be seen through the body-wall on the ventral side of the parapodia, particularly in nearly mature worms in which the body-wall is partly histolysed and is thinner than in the immature worms. Methylene-blue solution injected by micrometer syringe into a segment frequently passes immediately to the rest of the body, but occasionally it remains localized and observations can then be made on the movement of coelomic fluid when the proboscis is everted. If the worm everts its proboscis after being injected in the anterior segments, the methylene blue does not reach segments posterior to the 35th until after several eversions or after wriggling by the worm. Similarly, methylene blue injected into the middle segments of the body does not appear in the anterior segments when the proboscis is everted. Chapman (1951) has found that there is no increase in the hydrostatic pressure in segments in the middle region of the body during eversion of the proboscis, but that occasionally increases can be detected in the anterior segments. Examples of such increases of pressure are from 8 to 13 cm, from 21 to 24 cm, and from 5 to 15 cm sea-water (Chapman, 1951). Evidently the first few septa are able to isolate the proboscis region from the rest of the body. That the proboscis depends upon the increase in hydrostatic pressure in the anterior coelom can be demonstrated by inserting an open cannula in the coelom of the proboscidial region. This prevents the worm from everting its proboscis, and coelomic fluid is driven through the cannula when it attempts to do so.

Inversion of the proboscis is accomplished by the contractions of the proboscis retractor muscles and the relaxation of the longitudinal muscles. The worm elongates and the segments return to their normal resting volume and shape. Segments 35 to 45 become less dilated, but a hold is still maintained on the sides of the burrow by segments 15 to 35, which are now dilated by the presence of the pharynx within them.

The final stage in the burrowing cycle is movement forwards into the cavity made in the sand by the proboscis. *Nephtys* appears to be unable to ambulate merely by movements of the parapodia, presumably because the extrinsic parapodial muscles are not well situated for this task and the acicular muscles are not provided with a sufficiently rigid insertion on the parapodial wall unless the body executes an undulatory movement. The anterior 15 segments are thrown into waves, exactly like the locomotory waves of the swimming worm. One result of this is that the parapodia of these narrow segments make contact with the sides of the burrow as they perform their power-stroke when the crest of the undulatory wave reaches them. The wider, successive segments become involved in this locomotory movement also, but segments posterior to the 50th remain passive throughout the burrowing cycle and are dragged forwards. When the prostomium reaches the end of the hole previously made by the proboscis, the entire burrowing cycle is repeated, starting again with the sudden eversion of the proboscis.

The method of burrowing employed by *Nephtys* differs from that of such worms as *Lumbricus* and *Arenicola* in which the mechanics of burrowing have been studied by Gray and Lissmann (1938), Chapman (1950), and Newell (1950), and by Wells (1944, 1948, 1954) and Chapman and Newell (1947), respectively. The most significant differences spring from the fact that the body of *Nephtys* tapers towards each end, does not have a circular cross-section, and contains no circular muscles. The worm can therefore anchor itself to the substratum by only a comparatively short region of the body and cannot execute peristaltic burrowing movements as both *Arenicola* and *Lumbricus* can. Instead of burrowing in a relatively steady and continuous way, as the earthworm does, *Nephtys* burrows in a series of convulsive jerks. A further advantage enjoyed by *Lumbricus* but denied to *Nephtys* is that since the advancing tip of the former is extended by the contraction of the circular muscles which reduce its diameter, the pressure it exerts against the substratum is magnified by being applied over a small area (Newell, 1950). *Nephtys* is a carnivore and has a large, muscular proboscis, so that the pressure it exerts against the substratum is applied over a wide area. *Arenicola* is in some respects intermediate between *Lumbricus* and *Nephtys* in its method of burrowing. Although it moves by peristaltic contractions of the body-wall, the proboscis is used, either to agitate and soften the sand into which the anterior segments are then thrust (Chapman and Newell, 1947), or to scrape away the sand in front of the advancing tip of the worm (Wells, 1948).

THE FUNCTION OF THE LIGAMENTS

Unfortunately it has not proved possible to investigate experimentally the function of the ligaments while the worm is burrowing or swimming. Incision of the body-wall, which is necessary for cutting the ligaments, is followed by local contraction of the body-wall muscles that seals off the wound and prevents the normal functioning of that part of the body. Chapman (1951) found it extremely difficult to measure the coelomic hydrostatic pressure in *Nephtys* because of the great activity of the worms, and it has proved totally impracticable to do so when they are burrowing through the sand or swimming. However, a number of tentative conclusions may be drawn from a consideration of the structure of the ligaments, their disposition, and the behaviour of the worms.

The ligaments are composed of bundles of alternating inextensible fibrils and elastic filaments linked to cross-membranes which are also elastic and which partition the ligaments. The mechanical consequences of such a structure are these.

1. Since the elastic elements constitute less than half the total length of the ligament, and the rest is inextensible, the extensibility of the ligament is less than it would be were it composed entirely of the elastic material.

2. As a corollary of this, the restoring force generated by stretching the ligament a given amount is greater than it would be were the ligament composed entirely of the elastic material. (This may be readily verified by considering the expression for Young's modulus, $E = \frac{fL}{adL}$, where f is the force, a the cross-sectional area of the fibril, and L its length.)

Thus the ligaments appear to be suited for resisting deformation while permitting a degree of flexibility to the system, and for providing a restoring force when they are elongated slightly, without necessarily being under tension when no external forces are applied to the system. These conditions would not be met by wholly elastic or wholly inextensible ligaments, and the existence of ligaments with this unique construction in *Nephtys* suggests that the body of the worm is exposed to forces not experienced by other animals.

To judge by the disposition of the ligaments in the body of the worm, they have the following functions:

1. The median longitudinal ligament protects the nerve-cord from undue stretching when the worm is elongated. This is a function sometimes performed by a supra-neural muscle, as in nereids (Defretin, 1949; Smith, 1957). There is no such muscle in *Nephtys*.

2. The ligaments running from the attachment nodes on the ventral nerve-cord to those in the intersegmental groove prevent the deformation of the intersegmental body-wall during changes of shape of the segment. The chief antagonists of the longitudinal muscles are the dorso-ventral muscles, and the only muscles inserted in the intersegmental groove are those of the septum, the slender diagonal muscle (12), and the proximal posterior para-

podial muscle (10). Some of the other extrinsic parapodial muscles may assist in this function, but they are inserted more distally in the parapodium and cannot serve to anchor this part of the body-wall. Several polychaetes have reduced circular muscles, though it is rare for them to be lacking altogether as they are in *Nephtys*, and it is significant that the intersegmental body-wall of nereids, in which the circular muscles are slight, is strengthened by circumferential fibres (Smith, 1957). We do not know whether *Nephtys* possesses similar fibres, but it is evident that in nereidiform polychaetes with a reduced circular muscle-layer, there is a need for additional support in the intersegmental region of the body-wall.

3. In worms in which changes of shape depend upon a hydrostatic skeleton, it is important that pressure changes produced by one set of body-wall muscles should be applied to the antagonistic set of muscles and not dissipated by the dilatation of other parts of the body-wall. None of the extrinsic parapodial muscles of *Nephtys* runs to the tip of the parapodium, an unusual situation in nereidiform polychaetes, and the ligaments inserted in the acicular and chaetel sacs may supplement the acicular muscles and the intrinsic parapodial muscles in preventing the protrusion of the parapodium when the body-wall muscles contract. The lateral longitudinal ligaments are disposed in such a way as to prevent dilatation of the base of the parapodium.

A possible consequence of the method of burrowing is that the impact of the proboscis against the substratum produces a high, transient, fluid pressure in the coelom of the worm. The peculiar elastic properties of the fibres, and their disposition to support the weakest parts of the body-wall and to brace the septa from the anterior side, are particularly suited to resist sudden shocks of this kind. While many other polychaetes burrow in sand, and some may use similar methods of burrowing to those employed by *Nephtys*, they nearly all inhabit permanent burrows. *Nephtys* is one of the few polychaetes that is able to live in shifting sands; it does not form a consolidated, permanent burrow, and it is predaceous. There is every likelihood therefore that burrowing is a much more frequent activity of this worm than of other worms living in sandy beaches and this may account for its unique structures.

We are greatly indebted to Professor G. Chapman for permitting us to quote some of his unpublished data on the mechanism of proboscis eversion in *Nephtys*. His (earlier) account of the method by which the proboscis is everted agrees with our own.

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The Blood-cells of the Ascidian, *Phallusia mammillata*

By R. ENDEAN

(*From the Department of Zoology, University of Queensland*)

With one plate (fig. 8)

SUMMARY

The structure, histochemistry, and histogenesis of the blood-cells of *Phallusia mammillata* (Cuvier) have been investigated.

Primitive lymphocytes give rise, by a series of transition stages, to vanadocytes, pigment cells, phagocytes, and cells with acidic vacuoles. There is evidence that most of these cells must eventually leave the blood-stream. During the development of each cell type, RNA appears to be released from the nucleus, and there is a progressive reduction in nuclear volume.

Ninety-eight per cent of the total number of blood-cells are vanadocytes or their precursors. Each vanadocyte contains a number of globules. Each globule has a central core enclosed by a surface membrane containing compound lipid. In the core, a vanadium compound is associated with H_2SO_4, protein, and carbohydrate material, which may be the precursor of the tunicin of the test. Part of the carbohydrate material is in a dispersed form and can be hydrolysed with 2N H_2SO_4. The remainder is in the form of birefringent grains which resist hydrolysis, and appears to be a very insoluble polysaccharide.

Those lymphocytes that are destined to form vanadocytes develop vacuoles in their cytoplasm initially. In these vacuoles, which are ringed with granules that are probably mitochondria, granular material capable of reducing OsO_4 and $AgNO_3$ is formed. The vacuoles in each cell then unite to form one large vacuole, and refractile granules appear in the perinuclear cytoplasm. These granules then move out from the nucleus in the cytoplasm peripheral to the vacuole. Each refractile granule is then enclosed in a subspherical compartment in which protein material is formed. As the compartments are transformed into the globules of vanadocytes, acid, PAS-positive material, and a vanadium chromogen with reducing properties appear.

The pigment cells contain melanin and oriented submicroscopic crystallites.

INTRODUCTION

THE blood of ascidians has been studied by numerous investigators. For an introduction to the extensive literature on the subject the reader is referred to papers by George, 1939, Pérès, 1943, and Webb, 1956. A great diversity of blood-cells has been described, but little is known about their biochemical or physiological roles.

Perhaps the most interesting of the earlier discoveries was the finding by Henze (1911, 1912, 1913*a*, 1913*b*) of a vanadium compound associated with sulphuric acid, in certain morula-shaped corpuscles present in the blood of *Phallusia mammillata*. Cells of this type were subsequently termed vanadocytes by Webb (1939). In an Australian ascidian, *Pyura stolonifera* (Heller), cells similar to vanadocytes in many respects, but containing an iron compound instead of a vanadium compound, have been found (Endean, 1953,

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1955*a*). These cells participate in the formation of the test of this species, which contains tunicin (Endean, 1955*b*, 1955*c*).

The present work was initiated to find whether the vanadocytes of *Phallusia mammillata* also participate in test-formation. However, as several types of blood-cells were found in the test, it became apparent that a knowledge of the structure, histogenesis, and chemical constitution of all types of blood-cells present in this species was desirable. In this paper attention is focused on these aspects.

MATERIAL AND METHODS

Adult specimens of *P. mammillata* (Cuvier) were obtained from Salcombe, South Devon. Before use, they were kept in well-aerated sea-water.

The test in the vicinity of the heart of each specimen was removed and blood was withdrawn from the heart by means of a hypodermic syringe. About 5 ml of blood could be readily obtained from adult specimens of average size. Provided the syringe was perfectly clean and dry, and provided that air was not bubbled through the blood, clotting did not occur.

When required separately, plasma and corpuscles were separated by centrifuging at 2,500 r.p.m.

Living cells were examined in hanging drop preparations. In some cases they were dyed with supervital dyes (neutral red, methylene blue, Janus green B—each at a concentration of 0·001% in sea-water).

Fixed cells for cytological and cytochemical examinations were prepared as follows. A drop of blood was smeared over the surface of each slide. After 5 min, excess fluid was drained off, and each slide was flooded with 5% formalin in sea-water. After a 15-min interval, the fixative was drained off and the slides flooded with distilled water. This was drained off after 3 min and the smears allowed to dry.

As a result of this procedure, the fluid contents of some of the cells with large vacuoles tended to be released. Otherwise, cellular structures appeared intact, and a representative proportion of fixed cells adhered to the slides.

Alternatively, cells were exposed to the vapour of osmium tetroxide. Cytological structures were very well preserved by this fixative but one type of cell was completely blackened.

Weigert's haematoxylin, eosin, Mann's methyl blue / eosin, and 0·1% toluidine blue were used in general cytological studies.

Millon's reagent, prepared after the method of Bensley and Gersh (1933), and $HgCl_2$ / bromphenol blue (Mazia, Brewer, and Alfert, 1953) were used for the detection of proteins. For lipids, Sudan IV, Sudan black B, and burnt Sudan black B (Berenbaum, 1958) were used. Polysaccharides were sought by utilizing the PAS reaction (Hotchkiss, 1948; McManus, 1948). The localization of DNA was followed by utilizing the Feulgen reaction, and that of RNA by using 0·001% ribonuclease in conjunction with Unna's methyl green / pyronin mixture. The distribution of melanin was investigated by tilizing Fontana's ammoniacal $AgNO_3$ solution and by bleaching with 0·3% MnO_4 followed by 0·3% H_2SO_4 (Chesterman and Leach, 1958).

Small pieces of fresh test containing a blood-vessel were fixed in buffered % OsO_4 (Palade, 1952), dehydrated in 70%, 90%, and absolute alcohol, mbedded in araldite (Glauert and Glauert, 1958), and sectioned on an ltramicrotome of the A. F. Huxley pattern. Sections showing gold inter- erence colours were selected, mounted on grids coated with formvar, and xamined by means of a Phillips electron microscope.

RESULTS

he blood-corpuscles

Haemocytometer counts on blood-samples from adult specimens showed hat the average number of blood-cells per cubic millimetre of blood approxi- ates 68,000.

On the basis of differences in structure and of behaviour towards various yes and histochemical reagents, the following cell types were recognized: ymphocytes, phagocytes, amoebocytes with vacuoles, signet-ring cells, com- artment cells, vanadocytes, cells with acidic vacuoles, cells with reflecting isks, and pigment cells. However, this classification is a purely arbitrary one, nd some of these cell types represent stages in the development of others.

Lymphocytes. These small, spherical cells (fig. 1, A), which average approxi- ately 4·5 μ in diameter, are rare in the blood. Each possesses a large nucleus about 3·5 μ in diameter) and a small amount of finely granulated cytoplasm.

Mesh-like aggregations of basiphil material were apparent in the nuclei of rmalin-fixed cells. This material was blackened by burnt Sudan black B.

The cytoplasm was lightly basiphil and was coloured a faint blue by $IgCl_2$ / bromphenol blue. Pyronin dyed the cytoplasm in the immediate icinity of the nucleus. After treatment with ribonuclease the cytoplasm was ot dyed with pyronin.

No detectable PAS reaction was given by lymphocytes and no material olourable by Sudan black B was found in the cytoplasm.

Phagocytes. These cells (fig. 1, C) are characterized by their possession of lamentous pseudopodia, a prominent nucleus (averaging 3·2 μ in diameter), nd a variable number of minute granules in the cytoplasm near the nucleus. ccasionally, a few large granules are found elsewhere in the otherwise opti- ally homogeneous cytoplasm, which is relatively extensive compared with at of most types of blood-cell.

Carmine particles were introduced into the blood of living specimens of . *mammillata* by injecting 0·5 ml of finely ground carmine suspension in a-water into the heart by means of a hypodermic syringe. Eighteen hours ubsequently, blood-samples were removed from each specimen. Cells of the pe described above were packed with carmine particles and it was on this asis that they were considered to have a phagocytic function. Such phago- ytes comprised about 1% of the total number of blood-cells.

Neutral red coloured some of the cytoplasmic granules near the nucleus. Methylene blue coloured granules in a similar position.

Most of the cytoplasmic granules in formalin-fixed phagocytes gave a positive reaction for protein with $HgCl_2$ / bromphenol blue and some were PAS-positive.

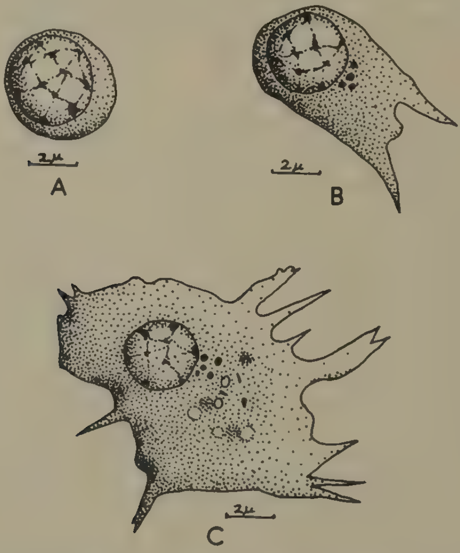


FIG. 1. A, lymphocyte. B, lymphocyte with pseudopods and increased cytoplasmic volume. C, phagocyte. All figures show cells fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin.

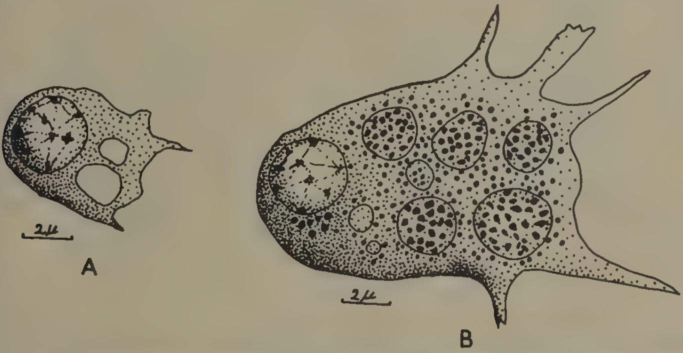
The general cytoplasm was lightly basiphil. The cytoplasm near the nucleus was dyed faintly by pyronin but not after prior treatment with ribonuclease.

Osmium tetroxide did not noticeably darken any constituent of these cells.

What appear to be transition stages between lymphocytes and phagocytes have been recognized in the blood. It seems that this transition results from a gradual increase in the cytoplasm of lymphocytes. At an early stage in this transition (fig. 1, B), filamentous pseudopodia are extended by the developing cells, and cytoplasmic granules became apparent near the nuclei.

Amoebocytes with vacuoles. About 6% of the total number of blood-cells are of this type (fig. 2, B). Each possesses a spherical nucleus (about 3·2μ in diameter) and a finely granulated cytoplasm, in which a variable number of roughly spherical vacuoles occur. A variable number of large granules is found near the nucleus. Pointed pseudopods are protruded by these cells.

Sometimes the vacuoles, which vary from 1 to 5μ in diameter, appear optically empty. Such vacuoles were coloured a faint orange by neutral red nd a pale blue by methylene blue; they were not darkened by exposure to smium tetroxide vapour. The majority of vacuoles, however, are finely ranular. These vacuoles were coloured strongly orange by neutral red, and lue by methylene blue. Exposure to osmium tetroxide vapour resulted in a reying of the vacuoles and a blackening of their contained granules.



IG. 2. A, small amoebocyte with two clear vacuoles. B, amoebocyte with vacuoles enclosing ranules. Both figures show cells fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin.

The large granules near the nucleus of the cells were coloured red by eutral red.

Janus green B revealed the presence of numerous small mitochondria, ome of which were scattered in the cytoplasm in the immediate vicinity of he nucleus of each cell, while others occurred between the vacuoles, sometimes ringing these vacuoles.

Much vacuolar activity was usually in evidence. Occasionally, one or more f the vacuoles merged. Sometimes new vacuoles were cut off from outocketings of pre-existing ones. The vacuolar granules, when present, were ndergoing Brownian movement.

The perinuclear cytoplasm was usually dyed faintly by pyronin, but not the cells had received prior treatment with ribonuclease.

Some amoebocytes with vacuoles took up carmine particles, though not s energetically as phagocytes.

PAS-positive granular material was usually in evidence near the nucleus. IgCl_2 / bromphenol blue coloured in blue both the intravacuolar granules nd the general cytoplasmic granules. These granules also gave a positive eaction for protein with Millon's reagent.

The cytoplasm was lightly basiphil.

Amoebocytes with one or more small, non-granular vacuoles (fig. 2, A), nd not much bigger than lymphocytes, were observed in the blood. As the uclei of these cells are approximately the same size as those of lymphocytes and have a similar distribution of chromatin, and as the cytoplasm of the two cell types is lightly basiphil, it is probable that these small amoebocytes with vacuoles arise directly from lymphocytes. Larger amoebocytes with vacuoles are formed as a result of a further increase in cytoplasmic volume and the formation of numerous vacuoles. However, the possibility that some of the larger amoebocytes with vacuoles arise as the result of the vacuolization of phagocytes should not be excluded. In either case, it would appear that there is a time interval between the formation of fluid-filled vacuoles and the appearance of intravacuolar osmiophil granules.

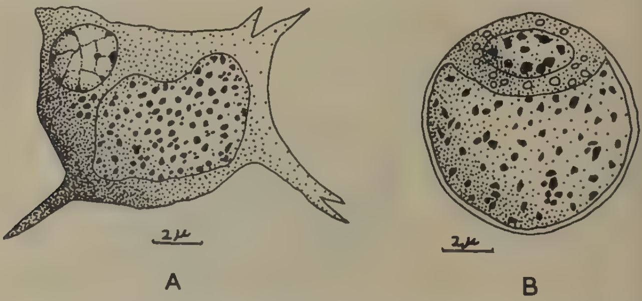


Fig. 3. A, amoebocyte with one large vacuole containing granules. B, signet-ring cell with refractile granules in perinuclear cytoplasm. Both figures show cells fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin.

Signet-ring cells. Typically, each of these approximately spherical cells contains a large fluid-filled vacuole in which granules showing Brownian movement are apparent, and a nucleus which is peripheral in position and surrounded by a small amount of cytoplasm. Fully developed signet-ring cells have diameters between 8 and 9·5 μ. When the nuclei of such cells are spherical, they average about 3 μ in diameter, but usually they are elliptica (fig. 3, B). Signet-ring cells constitute about 5% of the total number o blood-cells.

In some cells, but not all, greenish refractile granules occur in the cytoplasm near the nucleus. These granules were dyed blue by methylene blue and by Janus green B.

The intravacuolar fluid was coloured orange by neutral red. When exposed to osmium tetroxide vapour the fluid became greyish and the granules present in this fluid, black. Ammoniacal $AgNO_3$ darkened these granules in formalin-fixed signet-ring cells and caused the rest of the vacuolar content to assume a reddish-brown colour.

Both the granules in the perinuclear cytoplasm and the intravacuola granules of formalin-fixed cells were positive for protein with $HgCl_2$ / brom phenol blue reagent and with Millon's reagent.

No lipid was detected either in the vacuole or in the cytoplasm. Materia hich was faintly PAS-positive occurred in the perinuclear cytoplasm. The erinuclear cytoplasm was faintly basiphil, and contained RNA.

Irregular black areas were observed in the nuclei of signet-ring cells after eatment with burnt Sudan black B.

Signet-ring cells arise from amoebocytes with vacuoles. The granulated acuoles of some of these amoebocytes were observed, in hanging drop reparations, to coalesce to form one large vacuole (fig. 3, A). Such cells ehaved in the same way towards the dyes and histochemical reagents used n this investigation as signet-ring cells which do not possess large refringent ranules in the perinuclear cytoplasm. These granules, which appear to be rge mitochondria, from their behaviour towards Janus green B, are formed uring the signet-ring stage of development.

Compartment cells. About 44% of the total number of blood-cells are esignated 'compartment cells'. These spherical cells average 8·5 μ in iameter. Each contains a large vacuole which varies in size from cell to cell. ranules are apparent in the fluid in the vacuole. A number (8 to 18 were ounted in different cells) of subspherical compartments, peripheral in osition, obscure the vacuole except at one pole of the cell (fig. 4, B). At the pposite pole an eccentrically placed nucleus, averaging about 2·7 μ in diameter, s found (fig. 4, D). Each compartment encloses a greenish refractile granule. n different compartments the diameters of the granules range from 0·6 to μ. Compartment cells usually settle on a slide with their nuclear poles ownwards.

When a drop of distilled water was added to a drop of blood, the compartments became spherical and swelled markedly. The larger of these spherical odies were situated near the nuclear pole.

After exposure to 1:200 NH_3 the compartments became spherical. Initially hese spherical bodies averaged about 2 μ in diameter but they swelled rapidly nd were usually released from their parent cells. Each released spherical ody possessed a pale greenish oval core, in which a refractile granule was mbedded at a point at the periphery (fig. 4, C). The core was surrounded by thin film of clear material which was bounded by a membrane. Neither the ores nor the refractile granules were birefringent.

With dilute neutral red most compartments assumed an orange colour but he compartments of those cells which possessed smaller vacuoles than the ajority were coloured pink. With dilute methylene blue most of the compartments were coloured a pale blue, but the enclosed refractile granules oloured more strongly. Janus green B coloured these granules blue.

Osmium tetroxide vapour caused a greying of the boundaries and cores of he compartments and a blackening of the granular contents of the vacuoles f compartment cells.

When formalin-fixed cells were ruptured by the application of pressure to he coverslip covering them, compartments were released as angular subspherical bodies, the more spherical of these bodies being situated near the uclear pole.

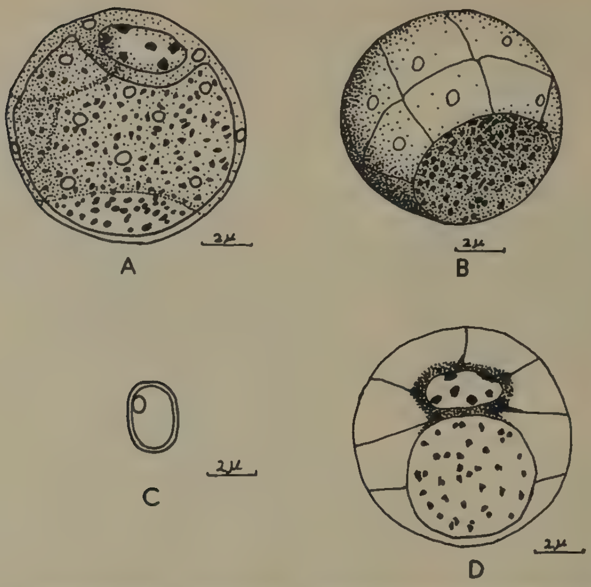


FIG. 4. A, a transition stage in development of compartment cell from signet-ring cell. The cell has been fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin. The cytoplasm enclosing the vacuole has begun to thicken and compartments are forming. B, a compartment cell fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin. C, drawing of isolated compartment fixed with OsO_4 vapour. D, semi-diagrammatic drawing of a compartment cell in optical section.

Formalin-fixed cells were treated with dyes and subjected to histological techniques as outlined in table 1.

Observations made on signet-ring cells and compartment cells kept in hanging drop suspension and dyed supervitally with methylene blue, revealed a series of transition stages. The refractile granules which appeared in the perinuclear cytoplasm of each signet-ring cell moved away from the nucleus and became lodged in the peripheral cytoplasm surrounding the vacuole of the signet-ring cells. Next, the cytoplasm near the nuclear pole of each cell thickened, and granular material appeared in the cytoplasm in this region (fig. 4, A). This granular material became aggregated to form, or mark the position of, the boundaries of compartments which formed first at the nuclear pole. Each compartment enclosed one of the large refractile granules. The inner border of each compartment formed a portion of the boundary of the large vacuole present in each cell (fig. 4, D).

Vanadocytes. Although their numbers fluctuate somewhat from specimen to specimen, these cells usually constitute about 43% of the total number of blood-cells. They are spherical in outline in fresh blood and contain

TABLE 1

Dye or reagent used	*Result*
osin	Most compartments dyed strongly
Veigert's haematoxylin	Nuclei dyed, but not strongly
lann's methyl blue/eosin	Nuclei dyed blue. Compartments of cells with large vacuoles dyed blue. Those of cells with small vacuoles dyed red
ontana's ammoniacal $AgNO_3$	Cores of compartments greyed. Vacuoles became reddish-brown with dark granules
AS reaction	A faint but positive PAS reaction given by the compartments of some small-vacuolated cells
lcoholic Sudan black B	No detectable coloration observed
urnt Sudan black B	The boundaries of compartments became faint brown. The refractile granules darkened. Spherical black areas were apparent in nuclei
lillon's reagent	The compartments of cells with large vacuoles became faintly orange, while the compartments of those with small vacuoles coloured more intensely
$IgCl_2$ / bromphenol blue	The compartments of cells with large vacuoles became a faint blue, whilst those of cells with small vacuoles coloured a deep blue
$IgCl_2$ / bromphenol blue after ribonuclease	A slight reduction in intensity of colouring occurred
yronin	The perinuclear cytoplasm was dyed strongly. The boundaries of compartments were coloured faintly
yronin after ribonuclease	No coloration occurred

number of greenish refractile globules encased in colourless cytoplasm ig. 5, A). On standing, or after fixation, vanadocytes assume a typical morula hape (fig. 5, C) and their globules become bluish. Such morulae have dia-ieters which range from 7 to 8·5 μ. Their contained globules vary in diameter om 2 to 3·5 μ.

When living vanadocytes were examined in hanging drops, little could be een of their internal structure, partially because of the way in which the lobules overlie one another, but chiefly because of the refractiveness of the urface membranes of the globules. The addition of a drop of distilled water a drop of blood caused the vanadocytes to swell and their globules, hitherto utually compressed, often separated from one another. Gentle pressure on e coverslip covering such a drop of diluted blood often resulted in the release globules from their parent cells. Eight to 28 globules were counted in ifferent cells.

On the addition of dilute (1:200) ammonia solution, vanadocytes became rown. This reagent also caused the refractiveness of the globules to be duced markedly, and frequently its use resulted in the release of many obules. Each such released globule possessed a thin, brownish, peripheral m surrounding a central core. The cores swelled considerably on standing in contact with dilute ammonia, and numerous granules and often one or several minute irregularly shaped grains could be observed within the cores (fig. 5, B). However, some vanadocytes did not possess such grains and in others only a few such grains were present. The irregularly shaped grains were birefringent. This was so particularly in the case of the larger grains, the more spherical of which were about 0·6 μ in diameter.

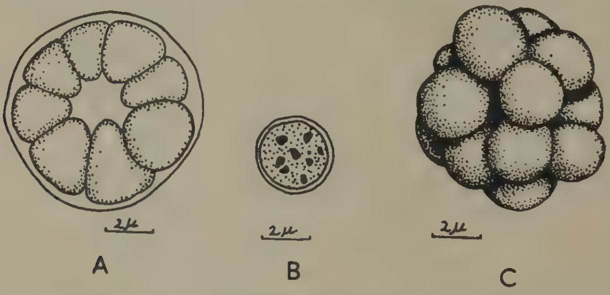


FIG. 5. A, a vanadocyte in optical section, dyed supervitally with neutral red. B, a globule isolated from a vanadocyte by treatment with dilute NH_3. C, a vanadocyte fixed with OsO_4 vapour.

Dilute methylene blue coloured the cores of the globules blue. Neutral red coloured them reddish, but there was much variation in the intensity of this colouring. Globules packed with birefringent grains were not coloured as intensely as others.

Treatment of vanadocytes with 0·5% acetic acid resulted in the gradual disappearance of the globules and their subsequent replacement by vacuoles. A nucleus (approximately 2·5 μ in diameter) could then be seen in each cell and extensions of the perinuclear cytoplasm formed the boundaries of the vacuoles.

Exposure to osmium tetroxide vapour resulted in the immediate blackening of the vanadocytes. Individual globules showed a black outer membrane and a dark grey core.

Cells fixed with formaldehyde turned blue on standing. After treatment with alcohol and xylene the refractiveness of the globular membranes was reduced and dark irregularly shaped birefringent grains could then be observed within most globules. Some globules were almost completely filled with these grains.

Cells fixed with formaldehyde were treated with dyes and histochemical reagents as outlined in table 2.

Cytolysis of vanadocytes in fresh blood-smears occurred on the addition of 70% alcohol. The greenish intraglobular material was observed to form a coagulum on each slide. This material was strongly PAS-positive and also contained protein, as evidenced by the blue coloration it assumed when the

TABLE 2

Dye or reagent used	*Result*
Weigert's haematoxylin	Globules dyed slightly, nucleus darkly
Eosin	No dyeing observed
Mann's methyl blue/eosin	In a few cases individual globules assumed a purplish colour. In cytolysed cells nuclei were coloured blue. In most cells no coloration was observed
Fontana's ammoniacal $AgNO_3$	Cores of most globules blackened immediately. In a few cases cores were dark brown and, rarely, golden brown. A thin peripheral film possessed by each globule did not colour
Millon's reagent	Globules became orange and the birefringent grains present in many globules appeared reddish
$HgCl_2$ / bromphenol blue	Most globules became yellow but a few were greenish. Birefringent grains did not change colour
PAS reaction	Globules gave positive reaction but there was much variation in the intensity of red colour produced from globule to globule. In some globules, the PAS-positive material was confined to the periphery of the globules, in others the whole globule was coloured, and in globules possessing birefringent grains, these grains and the material around them were positive
Salivary amylase followed by PAS reaction	Salivary amylase did not remove PAS-positive material
Toluidine blue	Core of globules coloured blue
Sudan IV	No visible reaction
Alcoholic Sudan black B	A faint blue coloration of surface membranes of globules
Burnt Sudan black B	Globules became brown, but brown colour appeared to be restricted to membranes of globules. The core and birefringent grains were no longer visible, but a black granule near the periphery of the core was evident in some cases
Pyronin	The globules were dyed reddish, but this coloration was confined to the periphery of the cores. The globules containing birefringent grains were not dyed as strongly as others
Pyronin after ribonuclease	Ribonuclease did not remove the material dyed by pyronin

$HgCl_2$ / bromphenol blue technique was used. The coagulum was not removed by N NaOH or 2 N H_2SO_4. After each of these reagents it remained positive for polysaccharide and protein.

Birefringent brownish grains were enmeshed in the coagulum and these gave a positive PAS reaction, but were not coloured by $HgCl_2$ / bromphenol blue. They were unaffected by N NaOH or 2N H_2SO_4.

Vanadocytes are formed from compartment cells as a result of the gradual obliteration of the vacuole of the compartment cell by increase in the volume of the compartments, and as a result of the compartments acquiring spherical shapes and refracting material on their surfaces. Compartments are thereby transformed into globules, and typical vanadocytes result.

During the transition from compartment cell to vanadocyte the nucleus is pushed from a peripheral to a more central position as a result of the rounding up of compartments peripheral to the nucleus.

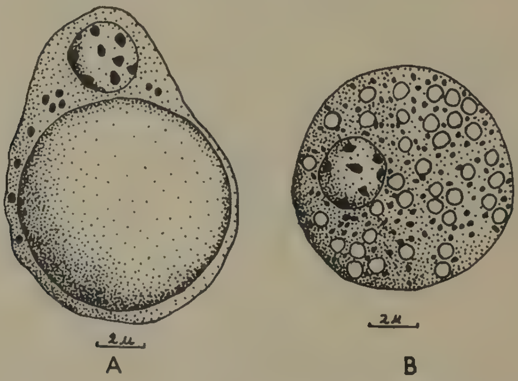


FIG. 6. A, a cell with an acidic vacuole, fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin. B, drawing of cell with reflecting disks, fixed with 5% formalin in sea-water and dyed with Mann's methyl blue / eosin.

The appearance of large numbers of birefringent grains within the globules of vanadocytes marks the end of the development of these cells in the blood.

The intraglobular material may be actively extruded from vanadocytes as evidenced by the following observations.

Many of the vanadocytes in hanging drops, which had been left to stand for 18 h, were observed under phase contrast to have ejected one or several (up to 7 counted) granules (about 1 μ in diameter), each of which was attached initially to a minutely 'beaded' thread to the parent cell. As the thread lengthened, the terminal granule diminished in size. Most of the extruded granules moved about actively and many of the longer threads were looped and twisted. Threads up to 30 μ long were observed.

Eventually, the threads broke away from the parent cells and many such isolated threads, still moving about vigorously, could be seen in each field.

On standing, many vanadocytes were observed to elongate and occasionally to produce blunt pseudopods.

Cells with acidic vacuoles

These cells are rare in the blood. Typically they possess a large fluid-filled vacuole (8 to 9 μ in diameter) and a spherical nucleus (about 3 μ in diameter); a few largish granules are scattered in the cytoplasm (fig. 6, A).

The vacuole was coloured red by neutral red and this dye also coloured the cytoplasmic granules. Methylene blue coloured the vacuole a deep blue.

Osmium tetroxide vapour greyed the vacuole but no osmiophil granules were evident in the intravacuolar fluid.

Basic dyes dyed the vacuole strongly but the cytoplasm was acidophil.

No PAS reaction was given by these cells.

The strongly acid nature of the vacuolar contents was attested by the yellow colour given by the $HgCl_2$ / bromphenol blue reagent.

A few small cells with vacuoles only 4 or 5 μ across, but exhibiting similar reactions towards dyes, have been observed in the blood. The nuclei of these cells were almost identical with those of lymphocytes, from which, presumably, they had arisen.

Cells with reflecting disks

Although always represented in blood-samples, these cells (fig. 6, B) comprise less than 1% of the total number of blood-cells. They are spherical, typically 9 to 11 μ in diameter, and possess a fluid cytoplasm which is packed with minute granules exhibiting Brownian movement. Numerous thin, disk-shaped structures (approximately 0·7 μ in diameter) which reflect light are also present in the cytoplasm. The nuclei (usually 2·5 μ in diameter) of these cells are always eccentric.

Neutral red and methylene blue did not colour these cells, but Janus green B dyed the granules blue.

The cells were not darkened by osmium tetroxide vapour or by ammoniacal $AgNO_3$.

The cytoplasm was strongly basiphil. It was dyed by pyronin, but not if the cells had received prior treatment with ribonuclease.

Cells fixed with formaldehyde gave a strong PAS reaction. The PAS-positive material was not removed by salivary amylase. Toluidine blue dyed the cells a deep violet.

The cytoplasmic granules were orange after treatment with Millon's reagent and were dyed blue by $HgCl_2$ / bromphenol blue.

Alcoholic Sudan black B coloured the cytoplasm a faint blue.

The reflecting disks were not dyed by any of the dyes used and gave no reaction with the histochemical reagents mentioned.

Cells as small as 5 μ in diameter and with only a few reflecting disks have been recognized in the blood. These cells had a granular and lightly basiphil cytoplasm. Their nuclei were about 3·1 μ in diameter. Such cells appear to be lymphocytes which have developed reflecting disks in their cytoplasm. Cells with reflecting disks and between 5 and 9 μ in diameter have also been observed in the blood. The nuclei of these cells range from 3 μ to 2·5 μ.

It would seem, therefore, that typical cells with reflecting disks develop from lymphocytes as the result of the formation of reflecting disks in the cytoplasm of lymphocytes and a gradual increase in cellular size with a concomitant decrease in nuclear size.

Pigment cells

A variable number (though usually less than 1% of the total number of blood-cells) of large disk-shaped cells (9 to 13 μ in diameter by 2 or 3 μ high) is found in the blood. Each cell (fig. 7, A) contains flat, yellow or orange bodies encased in a thin cytoplasmic envelope. From 7 to 18 of these coloured bodies occur in each cell. The largest observed was 12 μ long, 3·5 μ wide at the widest point, and 1 μ thick. The smallest was 5 μ long, 2·5 μ wide at the widest point, and 0·5 μ thick. The pigment bodies overlie one another with their long axes oriented in planes approximately parallel to the face of the disk-shaped cell.

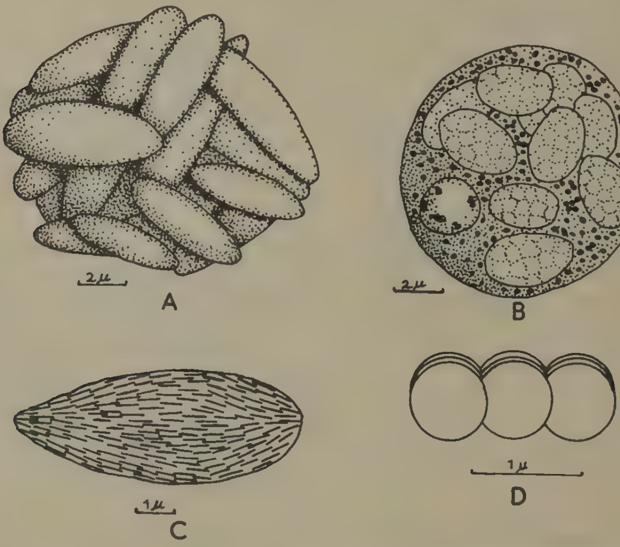


FIG. 7. A, a pigment cell fixed with OsO_4 vapour. B, drawing of a cell with developing pigment bodies. The cell has been fixed with 5% formalin in sea-water, dyed with Mann's methyl blue / eosin, and subjected to slight pressure. C, diagram of the apparent orientation of disks in a pigment body. D, diagram showing apparent orientation of 9 disks in a pigment body viewed in profile.

Under crossed nicols the pigment bodies showed strong birefringence. As noted by Webb (1939), the brightness of each was usually minimal when the long axes were oriented parallel to the plane of one of the nicols and maximal when inclined at an angle of 45° to each of them, but at no stage did the body extinguish as a whole.

Pigment cells were not coloured by neutral red, methylene blue, or Janus green.

Cells fixed with formaldehyde were not dyed by any of the dyes used in this

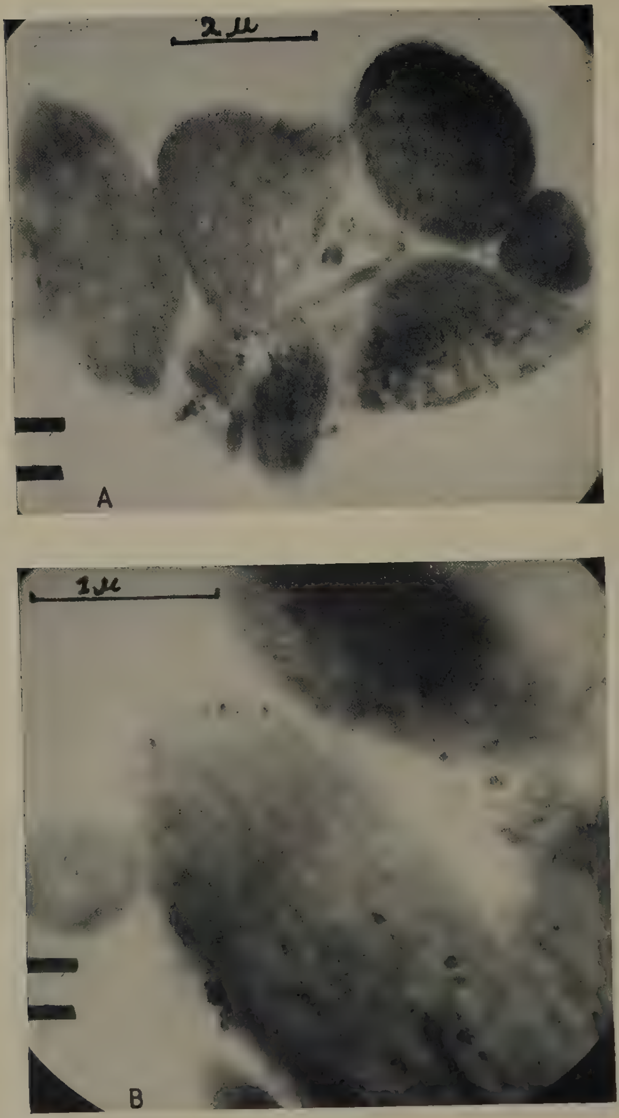


Fig. 8

R. ENDEAN

investigation. They were negative for protein, gave a negative PAS reaction, and were not coloured by alcoholic Sudan black B or burnt Sudan black B.

The coloured bodies swelled and became almost spherical when placed in distilled water. The pigment cells were resistant to the action of alcohol, acetone, glacial acetic, N HCl, and 2N H_2SO_4. Minute gas bubbles were, however, emitted from these cells for a short period when they were placed in contact with acid. N NaOH dissolved the pigmented material, and as the pigment dissolved the birefringence of the pigment bodies decreased and finally disappeared.

Treatment with a mixture of 0·3% $KMnO_4$ and 0·3% H_2SO_4 decolorized the pigment bodies, and they gave a positive argentaffin reaction. These facts indicate that the pigment is a melanin.

Fine pseudopods were sometimes put out by pigment cells.

Pigment cells are formed from cells with reflecting disks. In some of the latter cells the outlines of spherical (about 3μ) or slightly oval bodies were apparent amidst the oscillating granules. When such a cell was flattened by pressure on the coverslip, the outlines of reflecting disks could be seen inside the bodies (fig. 7, B). Such bodies were not dyed by any of the dyes used in this investigation. They were negative for protein, polysaccharide, and lipid. Subsequently the bodies became yellowish and reflecting disks could no longer be seen. The granules present in these cells disappeared gradually as the number of pigment bodies increased. Each pigment body acquired an elliptical shape as it developed and the nucleus was eventually hidden by overlapping pigment bodies.

Support for the presence of reflecting disks inside the pigment bodies was given by electron micrographs of pigment cells fixed in a blood-vessel of the test. Fig. 8, A, B shows sections of pigment bodies cut mainly in planes parallel to their long axes. In each pigment body, numerous rod-like structures about 40 $m\mu$ thick are revealed. Adjacent 'rods' are separated by material of higher electron density. Although the 'rods' belonging to a pigment body are all of approximately the same length, different pigment bodies possess rods ranging from about 0·3 to $0{\cdot}7\mu$ in length. The rods may represent disks cut in planes approximately at right angles to the flat planes of the disks and oriented as shown diagrammatically in fig. 7, C, D. As the thickness of the flat pigment bodies averages $0{\cdot}7\mu$ (which is the average diameter of the disks present in developing pigment cells and the length of the longer rods found in electron micrographs of the pigment bodies), the disks must be present only in a single layer in each pigment body. Fig. 8, B shows a body, circular in outline, and about $0{\cdot}7\mu$ in diameter. This probably represents a single disk which has been dislodged from a pigment body.

FIG. 8 (plate). A, electron micrograph of a section through a pigment cell, showing pigment granules and oriented crystallites in the pigment bodies.

B, electron micrograph of a section through pigment bodies. A disk-shaped crystallite, apparently dislodged from a pigment body, can be seen.

Small granules ranging from about 50 to 120 mμ in diameter are apparent in the pigment bodies. Similar granules are applied to the face of the disk in fig. 8, B. These granules show differences in density.

The nuclei of the blood-cells

Within the nuclei of lymphocytes, phagocytes, and some amoebocytes with vacuoles, the chromatin is dispersed in a meshwork. In the nuclei of many amoebocytes with vacuoles and all other cell types it occurs as a number of spherical bodies.

Nuclei fixed with methanol gave a positive Feulgen reaction and the DNA appeared concentrated in the regions just indicated.

With methyl green / pyronin the nuclei were usually dyed red, those of signet-ring cells, compartment cells, vanadocytes, cells with acidic vacuoles, and cells with reflecting disks strongly so. The nuclei of lymphocytes, phagocytes, and early amoebocytes with vacuoles were not dyed so strongly red and occasionally were dyed by the methyl green component of the dye mixture.

After treatment for 2 h with ribonuclease the nuclei were usually dyed only by the methyl green component.

Toluidine blue dyed the nuclei of all cell types violet and their behaviour towards this dye was not altered if the cells had received prior treatment with ribonuclease.

A faint but positive PAS reaction (not removed by salivary amylase) was given by the nuclei (and the perinuclear cytoplasm) of signet-ring cells, compartment cells, and vanadocytes. A stronger PAS reaction was given by the nuclei (and the general cytoplasm) of cells with reflecting disks.

Refractile bodies which were probably nucleoli were observed occasionally in the nuclei of lymphocytes but the great majority of this cell type, and all other cell types, were devoid of nucleoli.

Sugars in the blood-cells

Four lots, each of 10 ml of blood, were centrifuged, the plasma poured off, and the tubes allowed to drain. 4 ml of N NaOH was then added to each batch of packed corpuscles, which were broken up with a glass rod. A brownish solution (which turned blue if shaken with air, and which appears to be equivalent with the haemovanadin of Califano and Caselli, 1948) was obtained in each case. This was centrifuged off. A yellowish mass of aggregated material remained in each tube. Five millilitres of 2N H_2SO_4 were added to each tube and the contents agitated with a glass rod. The acid was then centrifuged off and the centrifugate in each tube washed with distilled water, alcohol, and ether.

The material from one tube was then examined microscopically. It consisted of amorphous, weakly birefringent material in which more strongly birefringent, irregularly shaped, brownish grains were enmeshed. The amorphous material behaved similarly to the coagulum formed by the intraglobular material from vanadocytes (p. 186) in being strongly PAS-positive and in reacting for protein with $HgCl_2$ / bromphenol blue. It was dyed a deep blue by toluidine blue, but gave no colour with burnt Sudan black B.

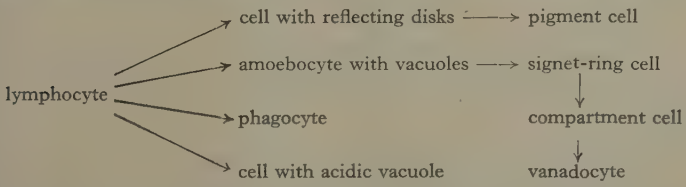
A positive PAS-reaction was given by the surfaces of the refractile grains but these were negative for protein and lipid when $HgCl_2$ / bromphenol blue and burnt Sudan black B respectively were used. The sizes, shapes, and birefringence given by these grains indicated that they were identical with the birefringent grains found in the globules of many vanadocytes.

To the material in each of the 3 remaining centrifuge tubes, 5 ml of 2N H_2SO_4 were added. Each acid mixture was then transferred to a separate boiling tube and heated at 100° C for 16 h. Brownish material still remaining in each tube at the end of this time was centrifuged off. It was found to consist of irregularly shaped, faintly birefringent brownish grains which were PAS-positive. These grains appeared to be grains from vanadocyte globules which had swollen and partially lost their birefringence as a result of treatment with acid. The hydrolysate from each tube was neutralized with $BaCO_3$. Insoluble material was removed by centrifugation and the supernatants concentrated by evaporation under reduced pressure. The concentrated supernatants gave a positive Molisch reaction, and reduced Benedict's solution.

Discussion

The lymphocytes of *P. mammillata* appear to give rise to all other types of blood-cells. No cells of the haemoblast type, believed by Pérès (1943) to be the primitive blood-cells of ascidians, were observed in the blood of this species.

A scheme of cell lineages consistent with the cytological and cytochemical findings recorded in this paper can be illustrated diagrammatically as follows:



The total number of blood-cells in undamaged specimens remains fairly constant and no marked disintegration of any cell type occurs in the blood. Also, the relative proportions of these cell types and of their various developmental stages remain constant. This indicates that there is a continual influx of lymphocytes (some of which may be already partially differentiated into other cell types) into the blood from some site or sites as yet undetermined, and an outflow of fully differentiated vanadocytes, pigment cells, phagocytes, and cells with acidic vacuoles. All these cells are capable of amoeboid progression.

As regards the structure and chemical constitution of vanadocytes, it has been shown that each vanadocyte globule possesses a surface membrane in which compound lipid is present. The membrane encloses a core which can be coloured by neutral red and methylene blue, which suggests that it is essentially a large vacuole. That this is so, can be established by viewing globules treated with dilute NH_3. The greenish fluid contents, in which there are granules, can then be seen. Also, the core swells when a globule is exposed to distilled water, and, when the globule bursts, a strongly acid, viscous fluid, containing granules, is released. The core contains no detectable lipid but contains protein and blackens with OsO_4 and $AgNO_3$. A vanadium chromogen is known to be present in vanadocytes (Henze, 1913*a*) and to be associated with protein (Califano and Caselli, 1948, 1950; Califano and Boeri, 1950; Bielig and others, 1954). The strong reducing properties of the chromogen would account for the blackening of the cores in the presence of OsO_4 and $AgNO_3$. PAS-positive material, unaffected by salivary amylase and not exhibiting metachromasy with toluidine blue, also occurs in the core. This material exists in a generally dispersed form in early globules; but later in development, PAS-positive birefringent grains are also present.

When released, the intraglobular contents form a coagulum which contains protein and is strongly PAS-positive. Birefringent grains which contain no protein but are PAS-positive are immersed in the coagulum. The coagulum and the grains are insoluble in dilute acid or alkali.

It was not possible to isolate vanadocytes for chemical analysis, from the other blood-cells. However, histochemical examination of the insoluble material remaining after blood-corpuscles from 10 ml of blood had been treated successively with acid, alkali, water, alcohol, and ether revealed that it contained protein and was PAS-positive. No lipid was detected in this material and it was not dyed metachromatically with toluidine blue. This suggests that the material consists of polysaccharide and protein, or glycoprotein. Part of the material could be hydrolysed with 2N H_2SO_4 and the hydrolysate gave a positive Molisch reaction and reduced Benedict's solution, which again suggests that the material contained carbohydrate. Of the cells present in 10 ml of blood, only vanadocytes could contribute a significant quantity of PAS-positive material.

The fraction not hydrolysed by 2N H_2SO_4 consists of the birefringent grains of vanadocytes. The grains did not give reactions for protein or lipid, and the fact that they were still PAS-positive after hydrolysis with 2N H_2SO_4 for 16 h indicates that they are composed of an insoluble polysaccharide.

During the development of vanadocytes there is a change in intracellular acidity. The vacuoles of amoebocytes with vacuoles, signet-ring cells, and compartment cells, and the compartments of early (i.e. large-vacuolated) compartment cells are approximately neutral in reaction. During the transition from compartment cell to vanadocyte there is an increase in acidity within the compartments. This is attested to by the increasingly red coloration given by neutral red during this transition and particularly by the change in coloration given by bromphenol blue. In the compartments of most compartment cells the colour of the indicator is blue. As these compartments are transformed into globules they first become green and subsequently are yellow. This would indicate an intraglobular acidity which is greater than pH 3. Henze (1911, 1912, 1913*a*, *b*) has shown that the acid is sulphuric acid, and Webb (1939) has estimated its concentration as approximately 9% H_2SO_4.

Material which blackens with OsO_4 and $AgNO_3$ first appears in the vacuoles of amoebocytes with vacuoles and is present in the vacuoles of signet-ring cells and compartment cells. If this material contains the vanadium chromogen, then the chromogen is formed in the absence of acid. However, a strongly acid medium is required if the chromogen is to be kept in the reduced state (Webb, 1939; Califano and Boeri, 1950). This suggests that the chromogen is not present in the vacuoles of the cells mentioned but possibly vanadium itself is accumulated initially in these vacuoles.

The chromogen, which has powerful reducing properties and turns blue when exposed to air, is present in vanadocyte globules which are formed from the compartments of compartment cells. Presumably, since the vacuoles of developing compartment cells gradually decrease in size and there is no concomitant increase in the concentration of material which blackens with OsO_4 and $AgNO_3$ within the vacuoles, this material passes into the compartments.

However, although acidophil protein is formed initially within the cores of compartments, no material which turns blue when exposed to air is present, and the cores only become faintly grey with OsO_4 and $AgNO_3$. Possibly the chromogen is present within the compartments but is in the oxidized state or in a form which will not reduce OsO_4 or $AgNO_3$.

It is noteworthy that the appearance in vanadocyte globules of acid, PAS-positive material and the vanadium chromogen with strong reducing properties coincide. This suggests that the chromogen is involved in the production of PAS-positive material, or in the production of acid, or in the production of both.

The present investigation has produced no evidence in support of George's (1939) suggestion that cells of the vanadocyte type act as nutritive cells for the tissues. It would appear that one of the chief functions of vanadocytes is the elaboration of polysaccharide material. The appearance of this material, in the form of birefringent grains, marks the end of vanadocyte development in the blood. This polysaccharide material may well be the precursor of the tunicin of the test. The ferrocytes of the ascidian *Pyura stolonifera* (Heller) participate in the production of test material, and ferrocytes are similar, in many respects, to vanadocytes (Endean, 1955 *a*, *b*, *c*).

Phagocytes, in the blood at least, do not appear to have any role other than a phagocytic one.

Cells with acidic vacuoles have no obvious role in the blood. Initially, these cells were thought to be aberrant signet-ring cells. Their strongly acid vacuoles, the apparent lack of granular material in these vacuoles, and the fact that the nuclei are not pushed against the peripheral cytoplasm, as they are in signet-ring cells, provide sufficient justification for making them a distinct cell type.

No function can be ascribed to pigment cells which confer on the blood its yellow colour. In the somewhat fluid cytoplasm of pigment-cell precursors, numerous small granules, and small disks which reflect light and appear to be inorganic crystallites, occur. Coincidentally with the formation of the precursors of pigment bodies there is a great reduction in the number of granules and disks discernible. The pigment itself appears gradually. Histochemical and solubility tests indicate that the pigment is a melanin.

Study of electron micrographs of pigment cells reveals the presence of structures which appear to be intercalating disks, arranged as in figs. 7, C, D. The average diameter of such disks corresponds with that of the disks recognized in developing pigment cells studied with the optical microscope. The arrangement of the disks in the ellipsoidal patterns revealed by the electron microscope is in accord with the conclusions of Webb (1939), who studied the birefringence of the pigment cells. Between the disks, granules showing differences in density are visible in some regions of the electron micrographs. These granules are probably melanin granules. They are similar in appearance to melanin granules in electron micrographs of cells of mouse melanoma S91 studied by Dalton and Felix (1953). The minute granules which dyed blue with Janus B in developing pigment cells may contain melanin precursors. Woods and others (1949) and du Buy and others (1949) found that melanized granules in melanoma cells were dyed with Janus green B.

Pigment cells with optical properties similar to those occurring in *Phallusia mammillata* have been found by Webb (1939) in *Ascidia mentula* Müller and in *A. conchilega* Müller. Pérès (1943) has found that the pigment in the orange blood cells of *Ciona intestinalis* (Linnaeus) is a melanin but in other ascidians carotenoids have been found (Azéma, 1929 *a*, *b*; Lederer, 1934; Webb, 1939).

The progressive decrease in nuclear size that occurs during the development of each principal cell type suggests that nuclear material may be utilized. As development proceeds, the chromatin becomes concentrated into a number of approximately spherical bodies. Histochemical tests show that DNA is present in the nuclei of all cells and that during development RNA appears in the nuclei and in the perinuclear cytoplasm. As the concentration of cytoplasmic RNA is greatest in the immediate vicinity of the nucleus, it is possible that RNA produced in the nuclei of developing cells diffuses thence into the cytoplasm. β-metachromasy with toluidine blue, indicative of the presence of compounds containing polymerized carbohydrate or phosphate (Pearse, 1953), was exhibited by the nuclei and perinuclear cytoplasm of developing cells. Part of the metachromasy exhibited may be due to RNA, which is weakly metachromatic (Baker, 1958), but most is not, as evidenced by the results obtained when the cells received treatment with ribonuclease before dyeing with toluidine blue. Since the nuclei and perinuclear cytoplasm of these cells give a weak PAS reaction and are positive for protein, mucoprotein or neutral mucopolysaccharide may be present. There is also the possibility to be considered that nucleoside-5-phosphates are released from the nucleus into the perinuclear cytoplasm. Adenosine-5-phosphate and adenosine triphosphate will give a PAS-positive reaction *in vitro* as noted by Pearse (1953).

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Cyclical Changes in the Distribution of the Testis Lipids of a Seasonal Mammal (*Talpa europaea*)

By B. LOFTS

(*From the Department of Zoology and Comparative Anatomy, St. Bartholomew's Medical College, University of London*)

With two plates (figs. 1 and 2)

SUMMARY

In the mole, *Talpa europaea*, there occurs a sequence of interstitial events comparable with that of seasonal birds, reptiles, and some fishes. There is a periodic accumulation and discharge of cholesterol and lipids in the Leydig cytoplasm. This cycle differs from its avian counterpart in that whereas in birds (and some fishes) the Leydig cells rapidly discharge their sudanophil contents before mating, the interstitium of the mole becomes exhausted much more slowly. Another difference is that the Leydig cells of the mole do not appear to become exhausted and replaced *en masse* as in birds after spermatogenesis. The seasonal growth and regression of the mammalian accessory sexual organs can be correlated with the increase and decrease of secretory activity of the interstitial cells (as shown by their lipid cycle). In the seminiferous tubules of the mole there is no lipid cycle corresponding to that observed in sub-mammalian vertebrates.

INTRODUCTION

As the seasonal spermatogenesis and sexual behaviour heightens in birds (Marshall, 1949, 1954), in at least one reptile (Marshall and Woolf, 1957), and in a fish (Lofts and Marshall, 1957), there is a progressive diminution of the Schultz-positive (i.e. cholesterol-containing) lipids in the endocrine cells of the testis, followed by their post-nuptial regeneration in preparation for the next sexual season. Further, after the shedding of spermatozoa, the residual contents of the seminiferous tubules undergo a massive steatogenesis accompanied by the appearance of quantities of cholesterol. Lofts and Marshall (1959) have shown that this material probably contains progestins.

Although such tubular lipids arise in mammals after hypophysectomy (Coombs and Marshall, 1956), their natural presence has not been shown in any mammal, although small amounts of lipid material arise (lipophanerosis) during normal mammalian spermatogenesis. Few, however, have been studied to this end (Wislocki, 1949).

The purpose of the present study was to examine the interstitium and seminiferous tubules of an emphatically seasonal mammal in order to discover whether cyclical events occur, comparable with those reported in sub-mammalian vertebrates.

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MATERIAL AND METHODS

It was considered that if tubule steatogenesis does occur in the Mammalia, the mole (*Talpa europaea*, Linn.), which undergoes great variation in testis size, might exhibit the phenomenon.

The seasonal changes in the genitalia of this animal have been studied in detail by several authors (Krasa, 1918; Courrier, 1927; Schwarz, 1928; Tandler and Grosz, 1912, 1913; and others). The majority of these workers, however, used traditional wax-embedding techniques which result in the dissolution of cytoplasmic and other lipids. Thus, many aspects of the testis cycle escaped notice.

The material for the present study consisted of 117 adult moles collected over a period of two years from January 1957 to October 1958 inclusive. Whenever possible the testes were dissected out and preserved within an hour after death, but in a number of cases (42) fixation was unavoidably delayed for periods of up to 24 h. The histological condition of the latter, however, did not appear to be much affected by post-mortem changes. After measurement of the whole gonad, testicular material was subjected to the following uniform procedures:

(1) One sliced organ was immersed in Bouin's fluid and subsequently embedded in wax. This material was sectioned at 6 μ and stained with iron haematoxylin and orange G for routine examination of spermatogenetic stages. (2) The remaining testis was fixed in formaldehyde-calcium solution, embedded in gelatine, sectioned at 8 μ on the freezing microtome, coloured with Sudan black, and stained with haemalum for the investigation of tubule and interstitial lipids. (3) Sections were subjected to the Schultz test for the detection of cholesterol. All measurements were made on the material embedded in wax.

RESULTS

Seasonal variations in size

The breeding season is short and is accompanied by great changes in the reproductive anatomy of both sexes, including a pronounced testicular expansion similar to that of birds and fishes. Table 1 shows that during March and April the gonads are at their maximum size of 17 × 10 mm, and subsequently undergo a very rapid regression to a minimum of 5·5 × 4·5 in July. There now ensues a period of inactivity during which the testes remain small. In January they again become active and expand rapidly throughout February.

Seasonal histological changes

January. The testis tunic is about 50 μ thick and the regressed tubules measure 80 to 90 μ in diameter. The tubules contain only spermatogonia (fig. 1, A) and are without sudanophil material or cholesterol. The interstitium is extensive and consists of large, rounded, Leydig cells measuring 12 × 10 μ with a nucleus 2 μ in diameter. The majority of the Leydig cells are completely free from cytoplasmic lipids, but some isolated cases do occur where the Leydig cytoplasm contains a few minute lipid droplets which are negative to the Schultz test.

TABLE I

Seasonal variation in testis size of Talpa europaea

Month	*No. examined*	*Testis size* (mm)
January .	6	10×5·9
February .	11	13×7·9
March .	10	17×10
April . .	8	14·5×7
May . .	11	8×5
June . .	9	6·5×4
July . .	7	5·5×4·5
August .	12	8×6·5
September .	10	8×6
October .	8	8×6
November .	14	8×5
December .	11	8×5

February. Tubule expansion has begun and the diameter is now 140 μ. The stretched testis coat is reduced to a width of 40 μ. Spermatogenesis has begun and the tubules now contain primary and secondary spermatocytes. In the interstitium an increasing number of Leydig cells are accumulating Schultz-positive lipids in their cytoplasm (fig. 1, B). The tubules are lipid-free.

March. The tubules have continued to expand owing to the ripening products within, and are now 250 μ in diameter. The tunic is reduced to a thickness of 30 μ. Bunched spermatozoa now occur. Within the lumen of some of the tubules there is a sparse scattering of minute lipid droplets (similar to those described by Wislocki (1949) in the testes of autumn-breeding deer). This is the normal vertebrate process of lipophanerosis which occurs during the transformation of spermatids into spermatozoa and has nothing in common with the massive post-nuptual tubule steatogenesis that occurs in birds and fishes. The expanded tubules have dispersed the interstitium. The Leydig cells are more lipoidal and Schultz-positive but comparatively few are strongly sudanophil (fig. 1, C).

April. The histological condition is little changed from that of the previous month. The tunic width is 30 μ, and the tubules are 230 μ in diameter and contain spermatozoa. There has been a considerable increase in the number of lipoidal Leydig cells (fig. 1, D).

May. Specimens collected during this month fell into two well-defined groups.

(1) Six specimens had testes with a histological appearance similar to that of the animals caught in the preceding month. The tubules were 220 μ in diameter and contained masses of spermatozoa (figs. 2, A, B). The interstitial Leydig cells were extensively lipoidal and many contained dense masses of sudanophil material and cholesterol.

(2) Five moles had discharged their spermatozoa and, as a consequence, tubule diameter had become reduced to a mere 50 μ. Spermatogonia only were now present and the tubule lumina were devoid of sudanophil material and cholesterol. The interstitium still consisted of large, densely lipoidal and Schultz-positive Leydig cells (fig. 2, C).

June. The testes are still small with a shrivelled tunic, 20 μ thick. The seminiferous tubules are unchanged, being 48 μ in diameter and containing only a few spermatogonia. The interstitial Leydig cells are less heavily charged with Schultz-positive lipids.

July. A proliferation of fibroblasts is laying down a new testis tunic within the old weakened coat (fig. 2, D). This double tunic is 85 μ wide and surrounds tubules 60 μ in diameter. No post-nuptial lipids have arisen in the tubules. The only germinal elements now are a peripheral layer of spermatogonia which, in most tubules, is a single cell deep. The Leydig cytoplasm still retains a little lipid and cholesterol but is much less sudanophil than in the June specimens.

August. The tunic is now 50 μ wide and most of the old coat has disintegrated. The tubules remain unchanged at 60 μ diameter with no germinal cells in advance of spermatogonia. The Leydig cells have now lost most of their lipid and cholesterol.

September to December. The histological appearance of the testis remains constant. The organ is ensheathed in a single new tunic 50 μ thick and there is an extensive interstitium surrounding tubules 70 to 90 μ in diameter. These remain inactive with only a peripheral ring of spermatogonia. The Leydig cells are denuded of sudanophil material and are negative to the Schultz test.

DISCUSSION

The interstitial cycle

The interstitial cycle of the mole has been studied by several workers (Tandler and Grosz, 1912, 1913; Courrier, 1927; Schwarz, 1928), who have advanced a number of conflicting viewpoints. According to Courrier (1927) these cells remain fairly constant throughout the year, not varying conspicuously in number or size. Tandler and Grosz (1912, 1913), on the other hand, reported that they vary inversely with the activity of the adjacent

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FIG. 1 (plate). A, testis in early January, showing regressed inactive seminiferous tubules containing only a peripheral ring of spermatogonia. The interstitium is extensive and contains large Leydig cells in a non-secretory condition. Bouin fixation; 6 μ paraffin-wax section stained with iron haematoxylin and orange G.

B, testis in February. Spermatogenesis has begun and the expanded tubules contain primary spermatocytes as well as spermatogonia. Leydig cells are starting to accumulate Schultz-positive lipid droplets. Formaldehyde-calcium fixation, 8 μ gelatine section coloured with Sudan black and haemalum.

C, testis in March. Tubules contain spermatids, and tubule-expansion has constricted the interstitial tissue. Technique as in B.

D, testis in April. Tubules are at the height of spermatogenesis. The interstitial Leydig cells are now densely lipoidal and Schultz-positive. Technique as in B.

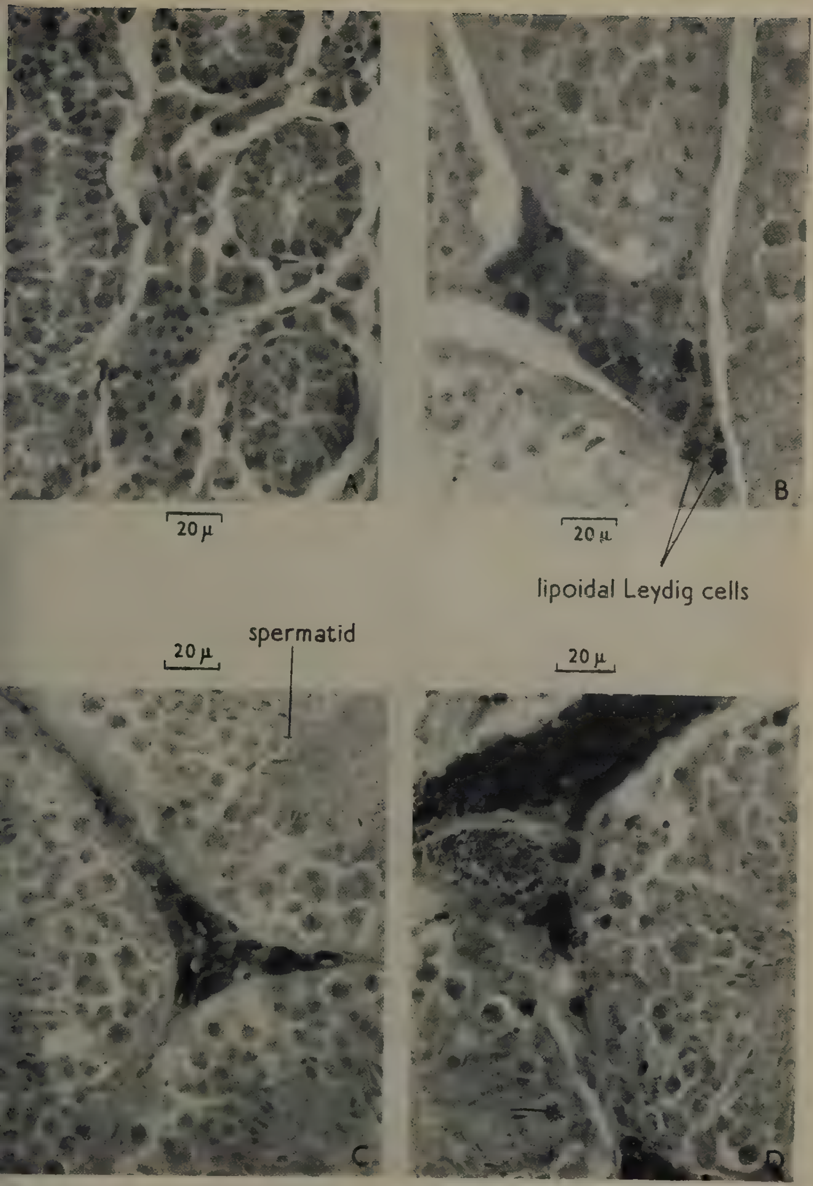

Fig. 1

B. LOFTS

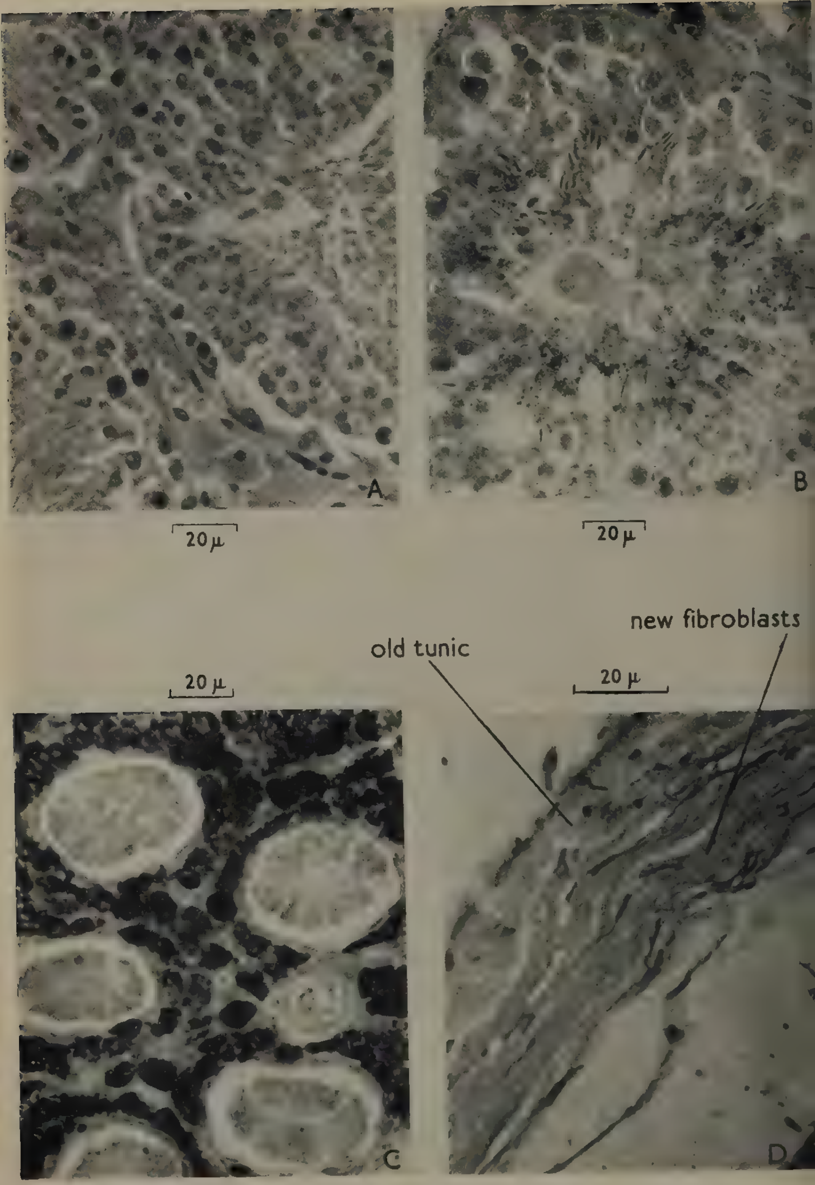

FIG. 2

B. LOFTS

seminiferous tubules, being smallest and least numerous during the period of maximum spermatogenesis and largest and most numerous during the anoestrous period. Further, Courrier also states that the interstitial cells contained rich cytoplasmic lipid all the year round. In my material this did not appear to occur.

The interstitium undergoes a well-defined cycle involving a gradual accumulation of lipid and cholesterol in the cytoplasm of the Leydig cells. This begins in January with the appearance of a few small sudanophil droplets in a few Leydig cells, and continues throughout the following months until nearly all the interstitium becomes involved and all Leydig cells contain dense Schultz-positive lipids. Maximum lipid content coincides with breeding activity and sperm-shedding in April and May. There now follows a gradual disappearance of sudanophil material from the interstitium, so that by late July to early August it is completely absent, and remains so until January.

This interstitial cycle of the mole is basically similar to that of birds in that it involves a seasonal accumulation and discharge of lipids and cholesterol. But whereas in birds and fishes the Leydig cells gradually discharge the sudanophil contents before mating, the interstitium of the mole becomes exhausted much more slowly and the testis still contains much Leydig lipid and cholesterol a month after sperm-shedding.

The testes of birds and the pike are almost never without lipid and cholesterol. During the immediate post-nuptial period, when a new generation of Leydig cells has arisen but has not yet accumulated sudanophil droplets, the neighbouring seminiferous elements have metamorphosed and contain large quantities of lipid and cholesterol in the tubule lumina (Marshall, 1949; Lofts and Marshall, 1957). While this is gradually clearing from the tubules through the winter months, sudanophil material begins to accumulate in the cytoplasm of the Leydig cells. The interstitium of the mole, on the other hand, has a period from September to December when it is devoid of lipid and cholesterol. Another point of difference is that in the mole the Leydig cells do not appear to become exhausted *en masse* and to be replaced by a new generation of juvenile cells, as occurs in birds after reproduction. The interstitium at all times appears to consist of rather large cells which remain more or less constant, apart from their lipid content, throughout the year. They appear somewhat larger and more numerous during the autumn and winter months because, during oestrous, the great expansion of the seminiferous tubules compresses

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FIG. 2 (plate). A, testis in April, showing tubules containing all stages of spermatogenesis including free spermatozoa. Technique as in fig. 1, A.

B, gelatine section of April testis, showing the fine lipid particles that are produced during apophanerosis. Technique as in fig. 1, B.

C, a 'spent' testis in the last week of May, showing great decrease in size of the tubules. These have discharged their spermatozoa and are without any sudanophil material. The interstitium still contains densely lipoidal and Schultz-positive Leydig cells. Technique as in g. 1, B.

D, part of the testis tunic in July. A proliferation of new fibroblasts is forming a new testis unic within the old, weakened coat of the regressed gonad. Technique as in fig. 1, A.

the interstitial tissue into tight wedges occupying the small interstices where several tubules meet, the cells between adjacent tissues becoming squeezed and compressed under the pressure of the ripening germinal elements within the lumina. It was probably this phenomenon that led Tandler and Grosz (1912, 1913) and Lecaillon (1909) to state that the interstitial cells showed their most marked development in the anoestrous period and decreased in number as spermatogenesis advanced. In fact, the present study has shown that interstitial activity is at its *highest* during the latter phase and this is supported by the parallel cycles of the accessory reproductive organs. Thus in March the vasa efferentia, epididymis, vasa deferentia, prostate, and Cowper's glands are greatly hypertrophied, and their epithelial elements are in a secretory condition (see Eckstein and Zuckerman, 1956). During the post-nuptial period the regression of these structures is rapid and closely follows the disappearance of Schultz-positive lipids from the cytoplasm of the Leydig cell cytoplasm, being complete by early July.

Tubule lipids

The only lipids observed in the tubules were minor amounts resulting from lipophanerosis, which is characteristic of vertebrate spermatogenesis. Very few mammals have been studied during every month of the year by techniques that would retain post-nuptial tubule lipids if they were in fact present. However, such do not occur in man (Montagna, 1952), vole (Marshall and Wilkinson, 1956), deer (Wislocki, 1949), nor, as has been demonstrated in the present study, in the European mole. These species represent a fairly wide selection of eutherian mammals. Monotremes and marsupials have not been studied in this connexion.

In the mole, and probably other seasonal mammals as well, part of the basic seasonal rhythm undoubtedly occurs. Thus, the interstitium undergoes a rhythmical lipid accumulation and depletion. However, at no time do the seminiferous elements undergo a metamorphosis involving the production of large masses of lipoidal material and cholesterol in the tubule lumina such as occur in some fishes (Lofts and Marshall, 1957), reptiles (Marshall and Woolf, 1957), and birds (Marshall, 1954).

Laboratory rats and mice, of course, show spermatogenetically active tubules all the year round. The interstitium is lipoidal and Schultz-positive throughout life. The seminiferous tubules at no season produce large quantities of sudanophil material or cholesterol, nor do prolonged and massive daily injections of prolactin cause tubular steatogenesis in mammals such as occurs in passerine birds (Lofts and Marshall, 1956). Yet hypophysectomy in rats leads to tubular steatogenesis (with the accompanying production of cholesterol), essentially similar to that which occurs naturally (and also after hypophysectomy) in birds. The interstitial cell tissue, however, does not regenerate as it does after removal of the anterior pituitary in domestic cockerels (Coombs and Marshall, 1956) and pigeons (Lofts and Marshall, 1958). Lacy and Rotblat (1958) have demonstrated that irradiation of rat testis also brings about a massive accumulation of Schultz-positive lipids in the tubule-lumina.

It is of phylogenetic interest that the testes of the Chelonia, Crocodilia, and Lacertilia contain Leydig cells with seasonally variable lipid content, and seminiferous tubules that undergo a post-spermatogenetic steatogenesis (Marshall and Woolf, 1957). In view of the common reptilian ancestry of birds and mammals, it seems possible that mammals too once possessed this tubular mechanism, which is still functional in seasonal birds. In mammals it has subsequently been lost as a naturally recurring phenomenon, though the mole, and no doubt many other seasonal mammals, still retain an interstitial cycle.

The testis tunic

As in birds, reptiles, and fish, a new testis tunic is manufactured seasonally by a resurgence of fibroblasts within the old wall. This phenomenon is probably common to most animals whose gonads undergo great seasonal enlargement. Such expansion, and the subsequent rapid contraction after spermatozoa are shed, weakens the testis coat, which then becomes replaced by a new tunic beneath, before the old one finally disrupts and disappears.

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A Method of Silvering the 'Golgi Apparatus' (Nissl Network) in Paraffin Sections of the Central Nervous System of Vertebrates

By G. B. DAVID, K. B. MALLION, *and* A. W. BROWN

(*From the Medical Research Council Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff*)

With two plates (figs. 1 and 2)

SUMMARY

1. It was accidentally found that methods of silvering synaptic end-feet sometimes blackened Golgi's 'internal reticular apparatus' in neurones of the central nervous system of the cat.

2. A method of achieving this consistently was worked out: (*a*) paraffin sections are coated with a collodion membrane; (*b*) the collodion membrane is soaked in silver nitrate; (*c*) the silver nitrate is reduced to metallic silver with a buffered formaldehyde solution; (*d*) steps (*b*) and (*c*) are repeated until the sections appear quite black; (*e*) the silver attached to structures other than the Golgi apparatus is removed with a ferricyanide/thiosulphate bleach; (*f*) the section is 'toned' with gold chloride, fixed in thiosulphate, and washed thoroughly; (*g*) the section is dehydrated, cleared, and finally mounted in Canada balsam, DPX, or similar media. *Results*: Golgi-apparatus, black; connective-tissue fibres, black; axons, grey to black; everything else is light grey or colourless.

3. A tentative hypothesis is advanced to explain the results obtained.

4. The following advantages are claimed for the new method: the cytoplasmic reticulum thus blackened resembles that seen in living neurones with the interference microscope; special methods of fixation are not required; the cytoplasmic reticulum of *given cells* can be studied before and after silvering; and serial sections of the same piece of tissue can be used for histochemical purposes.

INTRODUCTION

THE purpose of the work described here was to find a consistent method of silvering the 'Golgi apparatus' in paraffin sections of vertebrate nervous tissue. During a study of the synapse in the central nervous system of the cat (David, 1957; David, Brown, and Mallion, 1959), we found that our method of silvering synaptic end-feet sometimes blackened certain objects within the cytoplasm of neuronal perikarya (fig. 1). These objects resemble those seen by Golgi in the cytoplasm of the Purkinje neurones of the owl, and called by him 'internal reticular apparatus' (Golgi, 1898, especially his fig. 2). We then silvered a number of pieces of the spinal cord of a cat by one of the classical 'Golgi' techniques (Aoyama, 1929): similar objects were blackened in the cytoplasm of some of the neuronal perikarya. Therefore, it is convenient to call these objects 'Golgi apparatus'. Here the use of the term 'Golgi apparatus' does not imply a homology between the objects seen in the cytoplasm of vertebrate neurones (illustrated in Golgi's fig. 2 (1898) and our fig. 1) and the objects in other cells that have been referred to by the same name (see Cain, 1954).

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Very recently, Malhotra (1959) discovered that certain objects, essentially similar in structure to the Golgi apparatus, could be seen with the interference microscope in living neurones of birds and rodents. This discovery inspired us to look for similar structures in living neurones isolated from the ventral horns of the cervical enlargement of the spinal cord of the cat. We chose to work with these cells because we were already studying them for other purposes and because they were suitable objects with regard to size, ease of isolation, and uniformity of function. When the neurones were mounted in a saline solution (Baker, 1944), and were carefully examined with the 2-mm double-focus lens of the Smith interference microscope, a richly organized cytoplasmic structure could be seen. It was not possible to see this structure with any other method of vital examination. It seemed to us (David, Mallion, and Brown, 1959) that this structure, that described by Malhotra, and that blackened by silver in our sections, were one.

Since the objects under consideration were not artifacts and could legitimately be called Golgi apparatus, we set out to silver deliberately what before had been blackened by accident. This work was simplified by the fact that the appearance of successful preparations was known beforehand from our work with living cells. Eventually, a reasonably reliable technique was worked out. This method works with paraffin sections of material fixed and after-treated by routine methods.

FIXATION AND AFTER-TREATMENT

Two requirements must be met: (i) the Golgi apparatus must be present in the sections; (ii) no appreciable concentrations of extraneous substances capable of interfering with the process of silvering must remain in the sections.

The preservation of the Golgi apparatus is best verified by examining with the interference microscope those sections one intends to silver (fig. 2). Glycerol (r.i. = 1·473) is a suitable immersion medium. It is convenient to prepare permanent mounts of some of the sections processed in a given way, but not actually silvered. Kaiser's (1880) glycerol jelly is a suitable mounting medium for the purpose. Whether a given method of processing interferes with the silvering must be discovered empirically. It is customary to fix material to be silvered by the classical Golgi methods in aqueous solutions of formaldehyde containing substances that are supposed to facilitate the silvering, such as arsenious acid (Golgi, 1908) and salts of uranium (Ramón y Cajál, 1914), cobalt (Da Fano, 1920), or cadmium (Aoyama, 1929). Ethanol (Golgi, 1908) or methanol (Ramón y Cajál, 1914) are sometimes added to these solutions. We found that the Golgi apparatus could frequently be seen with

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FIG. 1 (plate). Optical sections through a neurone of the ventral horn of the cervical spinal cord of the cat, fixed in 'FAM', double-embedded, and cut at 10 μ; silvered by a method for end-feet (David, Brown, and Mallion, 1959).

A, photographed with the Golgi apparatus of the large dendrite on the right of the perikaryon in focus.

B, photographed with the plasmosome and parts of the nuclear membrane in focus.

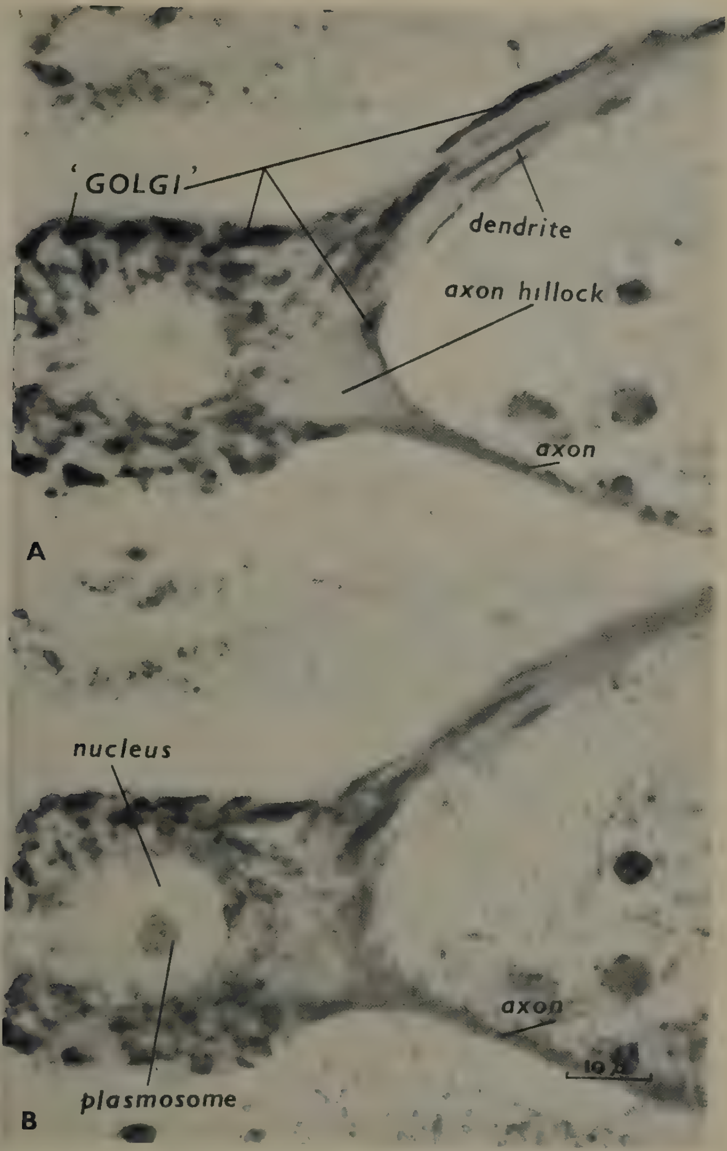


FIG. 1

G. B. DAVID, K. B. MALLION, *and* A. W. BROWN

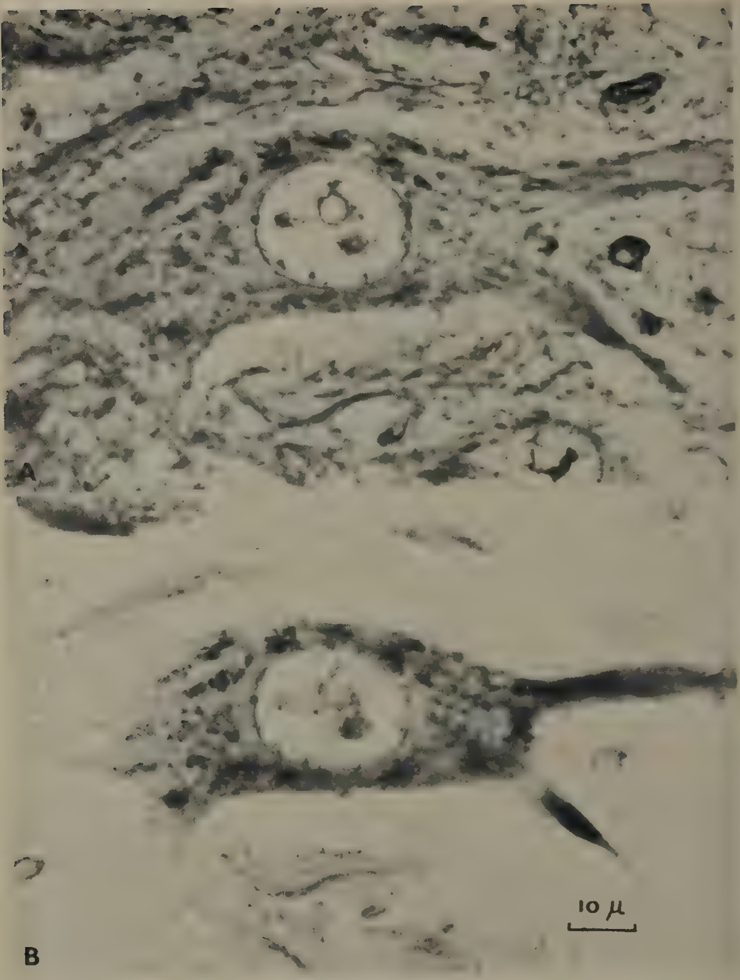


FIG. 2

G. B. DAVID, K. B. MALLION, *and* A. W. BROWN the interference microscope, and blackened with silver, in paraffin-sections of neurones of the spinal cord of the cat, that had been fixed in formaldehyde solutions containing only an isotonic concentration of sodium or calcium chloride. We tried the adjuvants given above and found that none appeared to affect the preservation or the silvering of the Golgi apparatus. Irrespective of the fixative used, objects similar to Golgi's (1898) fig. 2 could only be seen in some of the neurones. Since aqueous formaldehyde fixatives do not prevent severe shrinkage and distortion in the after-treatments required to produce paraffin sections, it is better to avoid them.

The most consistent results were obtained with material fixed in a reagent, 'FAM', introduced as a substitute for Carnoy's fluid (1887), when it is wished to fix nervous tissue for work on retrograde degeneration (David, 1955):

formaldehyde (undiluted commercial solution)	1 volume
glacial acetic acid	1 volume
absolute methanol	8 volumes

Methanol is used to coagulate proteins. It is preferable to ethanol, since it forms a finer coagulum; it diffuses more rapidly, but dehydrates more slowly, and therefore shrinks less. Acetic acid precipitates nucleoprotein and counteracts the shrinkage produced by the alcohol. Formaldehyde stabilizes proteins, ipoproteins, and proteolipids, and irreversibly inactivates ribonuclease. Formaldehyde, in 'FAM', does not fix tissues in the same way as it would in an aqueous solution. Since proteins of tissues fixed in 'FAM' do not become markedly basiphil and oxyphobe, it is unlikely that, in this instance, formaldehyde forms methylenic bridges between basic groups in peptide chains. Tissues fixed in 'FAM' fluoresce in ultra-violet light as formaldehyde polymers would. 'FAM' has a pH of 2·55, and will fix about 9 mm of cat-brain in 20 h (this corresponds to a *K*-value (Medawar, 1941) of 2·0). Small pieces of spinal cord are adequately fixed overnight at room temperature. After fixation in 'FAM', the pieces should be dehydrated in absolute methanol and double-embedded in collodion and paraffin. Pieces of tissue processed in this way are often of approximately the same volume as before fixation (David, 1955). It s a good plan to cut series of sections at different thicknesses. Details of the Golgi apparatus are best resolved in thin sections, of about 3 to 5 μ; the ramifications and general appearance of the network are best seen in 7- or even 10-μ sections. Unfortunately, it is extremely difficult to obtain good photomicrographs of the thicker sections. Double-embedded sections are mounted exactly as if they were ordinary paraffin sections. It is essential that the slides be thoroughly dry before silvering is attempted.

FIG. 2 (plate). Neurone of the ventral horn of the cervical spinal cord of the cat, fixed in FAM', double-embedded, and cut at 5 μ.

A, photographed with the interference microscope before silvering; mounted in glycerol.

B, photographed with direct illumination after silvering by the method described in this paper.

SILVERING

The silvering of tissues is based upon the reaction

$$\mathrm{Ag}^{+}+e^{-} \longrightarrow \mathrm{Ag}\downarrow \tag{1}$$

The whole problem is to ensure that metallic silver is attached only to those structures that one wishes to blacken. In the past hundred years, countless ways of doing this have been proposed. Techniques differing only in minor respects will blacken selectively neuronal perikarya, 'neurofibrils', synaptic end-feet, axons, different types of neuroglia, and connective-tissue fibres. Practically all methods of silvering are modifications of the original methods for axons, invented long ago by Golgi and Ramón y Cajál. It will be recalled that Golgi (1898) discovered his 'internal reticular apparatus' when he tried to blacken neuronal processes in pieces of nervous tissue that had been fixed too long. The mechanism of silvering axons has been studied in great detail by Liesegang (1911), Silver (1942), Holmes (1943), Nauta and Gygax (1951), Samuel (1953), Peters (1955), and Wolman (1955); the reader is referred to their papers for details. Methods of silvering axons comprise three basic steps: 'impregnation', 'reduction', and 'fixation'. Tissue is impregnated by soaking it in solutions containing silver ions (generally as silver nitrate, $\mathrm{Ag^{+}NO_3^{-}}$). Some of the silver combines chemically with the tissue—probably with terminal amino-acid residues of peptide chains—and some is attached to the tissue as minute silver nuclei: in neither case is an image formed. In reduction, appreciable quantities of metallic silver are produced when the tissue is treated with a reagent capable of supplying the electron required for the reaction given in equation (1) to proceed. This reaction is catalysed by the silver nuclei in the tissue. Finally, the silver is 'fixed' with sodium thiosulphate, $\mathrm{Na_2S_2O_3}$. In this step, any unreduced silver remaining in the tissue passes into solution as complex argyrothiosulphates (Mees, 1946, pp. 511–12):

$$\begin{aligned}\mathrm{Ag}^{+}+\mathrm{S_2O_3^{=}} &\rightleftharpoons \mathrm{AgS_2O_3^{-}}\\ \mathrm{AgS_2O_3^{-}}+\mathrm{S_2O_3^{=}} &\rightleftharpoons \mathrm{Ag(S_2O_3)_2^{\equiv}}\end{aligned} \tag{2}$$

Unfortunately, the mechanism of silvering structures other than axons has not been studied in detail. It has tacitly been assumed that the same basic reactions were involved, but there has been no proof of this. So far, no theory has been advanced to explain why certain structures are selectively blackened in some methods of silvering, and completely different structures, often containing completely different substances, in others.

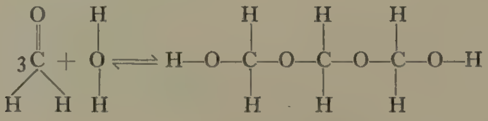
It occurred to us, when working on the synaptic end-feet, that differences in the argyrophilia of certain tissue-constituents, when silvered in certain ways, might be due to differences in the permeability of these tissue-constituents to silver that was reduced to particles of different sizes. We then developed a technique for silvering end-feet in which the tissue was not impregnated at all. Instead, ionic silver was reduced to particles of metallic silver of uniform size, which passed through a collodion membrane attached to the tissue (David, Brown, and Mallion, 1959). This technique had many features in common with the century-old photographic process known as the 'collodion wet-plate' (Archer, 1852). It was by this technique that we first blackened the Golgi apparatus accidentally. Then we studied the effects of modifying each step in this technique until we found that the Golgi apparatus could be blackened reliably. It is convenient to consider separately the following steps: (i) preparation of the collodion membrane; (ii) silvering and reduction; (iii) differentiation of the Golgi apparatus; (iv) finishing procedures.

(i) *Preparation of the collodion membrane.* This is the most delicate step of the procedure. The collodion membrane must be thin enough to allow the reagents used in the subsequent steps to penetrate readily, and it must be very uniform. We tried various concentrations of collodion (low-viscosity nitrocellulose, moistened with butanol, sold by G. T. Gurr, 136 New King's Road, London, S.W. 6), from 1 to 8% w/v, in the following solvents: ethanol / diethyl ether, methanol / diethyl ether (equal volumes); 'cellosolve' (ethylene glycol monomethyl ether), and amyl acetate. The best results were obtained with 1% w/v collodion in equal parts of ethanol and diethyl ether. To apply the collodion membrane, bring mounted sections to absolute ethanol after dissolving the paraffin in xylene. Take a slide from the jar of ethanol and blot it almost dry. Pick up the slide by one end and dip it vertically in a jar of the collodion solution. Move it about in the collodion for a few seconds. Withdraw it from the collodion *vertically*, steadying it with the left thumb and index finger. When the slide is out of the jar, grip its lower edge with the left hand and swing it rapidly through a wide arc. If the slide is not swung in this way, the membrane tends to be uneven. Now, put the slide in a jar of 70% ethanol and agitate it until diffusion currents disappear. Remove it and carefully wipe off the collodion except in the immediate vicinity of the sections. Rinse the slide in fresh 70% ethanol and store it in 50% ethanol. The time taken from the moment the slide is put in the collodion solution to the moment it is put in the first change of 70% ethanol should be between 15 and 20 sec. Longer times than these invariably result in collodion membranes that are either too thick or too impermeable for the purpose. We have not succeeded in obtaining continuous membranes with shorter times. It is convenient to coat several slides before proceeding further. Coated slides can be stored in 50% ethanol for several hours without affecting the final results.

(ii) *Silvering and reduction.* In the method for end-feet, we silvered the collodion membrane with an argentammine solution that consisted largely of silver diammine and silver monoammine, in equilibrium with silver, ammonium, and hydroxyl ions. With this, the Golgi apparatus can only be blackened occasionally. The most consistent results were obtained with a half-molar solution of silver nitrate in 50% methanol. The pH of this solution is 3·6, as is that of an aqueous solution of the same concentration. But aqueous solutions cannot be used, since metallic silver is reduced from these as coarse particles that cannot penetrate the collodion. The exact concentration of silver nitrate is not important—from about 0·2 to 0·5 M—but the pH should not be less acid than 3·8. The reason for this is that 'impregnation' (i.e. the formation of silver nuclei in the sections, and the chemical combination between the silver ions and the tissue) does not take place rapidly at acid levels (Peters, 1955*c*). When impregnation is allowed to proceed (for instance, when the membrane is silvered overnight), it is difficult to blacken the Golgi apparatus: the silvering then proceeds as in the methods for axons.

To silver the collodion membrane, place a coated slide on a staining rack, with the sections facing upwards. Flood the slide with the silvering solution (1 ml is ample), drain, and flood once more. One minute is long enough for the silvering, but no harm results from slightly longer periods.

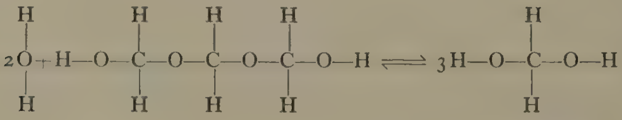
The next stage is reduction. This supplies the electron required for ionic silver to be reduced to metallic silver. Golgi (1908) reduced in a mixture of hydroquinone, formaldehyde, and sodium sulphite, and said that any 'common photographic developer' would do. We got more consistent results with formaldehyde alone. A simple aqueous solution of formaldehyde will not do. 'Formaldehyde solution' of analytical quality, as supplied to us, contains about 37% w/w formaldehyde, and has a pH of 3·1 (measured with the glass electrode). Its main impurity is formic acid, formed by the autocatalytic oxidation of formaldehyde (Walker, 1953). It is not a strong reducing agent. Formaldehyde gas in aqueous solution polymerizes reversibly, to form polyoxymethylene glycols; most of the formaldehyde in the commercial solution is found as trioxymethylene glycol:



$$3\,\mathrm{H_2C{=}O} + \mathrm{H_2O} \rightleftharpoons \mathrm{H{-}O{-}CH_2{-}O{-}CH_2{-}O{-}CH_2{-}O{-}H} \qquad (3)$$

(trioxymethylene glycol)

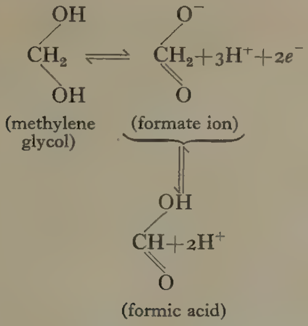
In dilute aqueous solution, trioxymethylene glycol depolymerizes to yield methylene glycol, the active monomer:



$$2\,\mathrm{H_2O} + \mathrm{H{-}O{-}CH_2{-}O{-}CH_2{-}O{-}CH_2{-}O{-}H} \rightleftharpoons 3\,\mathrm{H{-}O{-}CH_2{-}O{-}H} \qquad (4)$$

(methylene glycol)

The point of equilibrium of the reaction given in equation (4) can be shifted to the right by rendering the formaldehyde solution alkaline. In alkaline solutions, methylene glycol is readily oxidized, with the liberation of the electron required to produce metallic silver:



(5)

The reduction of silver nitrate then proceeds as follows:

$$2Ag^+NO_3^- + 2e^- \longrightarrow 2Ag\downarrow + 2NO_3^- \qquad (6)$$

We found it convenient to buffer an aqueous solution, containing 16% w/v formaldehyde, with 5·1 g borax ($Na_2B_4O_7 . 10H_2O$) per 500 ml; this has a pH of 8·5. Silver nitrate and borax react, to produce silver tetraborate and sodium nitrate. This side reaction slows down the precipitation of metallic silver, since a little silver is abstracted by the sodium tetraborate, and the pH of the solution drops rapidly. The metallic silver then comes down as particles of colloidal dimensions. If the reaction is carried out in a test tube, it can be seen that the silver thus formed is at first so fine that it only produces a slight, golden opalescence of the solution. If this is allowed to stand, progressively larger aggregates of silver become visible. The complete reactions between the silvering solution and the reducing fluid are as follows:

$$2AgNO_3 + CH_2(OH)_2 \longrightarrow 2Ag\downarrow + 2HNO_3 + HCOOH$$

$$2AgNO_3 + Na_2B_4O_7 . 10H_2O \longrightarrow \underset{\text{(silver tetraborate)}}{Ag_2B_4O_7 . 2H_2O\downarrow} + \underset{\text{(sodium nitrate)}}{2NaNO_3} + 8H_2O \qquad (7)$$

The insoluble silver tetraborate floats to the surface of the pool of liquid on the slide.

To start the reduction, drain the silvering solution from the slide, and replace it on the staining rack, *without washing*. Pour the formaldehyde reducer from a dropping bottle, using only enough of this to cover the sections (about 5 to 10 drops). Since the collodion membrane was soaked in an alcoholic solution of silver nitrate, turbulence can now be seen at the surface of the collodion membrane. This provides adequate agitation and ensures that the silver nitrate is reduced at the surface of the collodion membrane. Metallic silver, thus formed, reaches structures within the sections by a process of ultra-filtration (Ostwald, 1919). Obviously, only particles smaller than the pores of the collodion membrane can reach the section. The silver tetraborate formed in the secondary reaction given in equation (7) is carried to the surface of the fluid on the slide. As soon as the turbulence on the surface of the slide slows down (this takes about 30 sec), the reaction must be stopped. If this is not done, the silver tetraborate will be precipitated at random on the collodion membrane, and the particles of metallic silver within the membrane will increase in size to a point where the membrane is ruptured. The reaction is stopped by washing the slide for a minute or two under a direct jet of running water (tap-water is suitable). It is important to wash vigorously; the sections are not damaged by the jet of water, since they are protected by the collodion membrane. During the washing, the sections become perceptibly darker; this is probably due to the catalytic reduction of the ionic silver remaining within the membrane (Mees, 1946).

If the sections are now examined, preferably with a 3·6-mm water-immersion objective, the axons appear dark brown, the neuronal perikarya, golden; the Golgi apparatus is, at the most, golden reddish; the connective-tissue fibres are reddish brown; different types of neuroglia are light yellow to dark gold. We found that by repeating the silvering and reduction several times, the Golgi apparatus could eventually be blackened. Since metallic silver has a catalytic action upon the reduction of ionic silver, so that silver is deposited upon the silver already in the tissue, the blackening of those structures that remained colourless after the first silvering will only start when the structures that were silvered from the beginning become saturated with metallic silver. For this reason, the Golgi apparatus only begins to take up appreciable quantities of silver when the remaining structures in the section (with the exception of the cytoplasmic ground-substance) are almost black. If the permeability hypothesis to explain argyrophilia (given in p. 210) is correct, one would expect that structures not very permeable to silver—such as the Golgi apparatus—would resist de-silvering as much as they resisted silvering. Fortunately, this is so. For the Golgi apparatus to be successfully differentiated it is essential that it should be saturated with silver before bleaching is begun. To do so, it is necessary to repeat the silvering and reduction procedures from 3 to 5 times. It is convenient to examine the first slide of a batch after each silvering: if the conditions remain constant, the remaining slides will require the same number of treatments.

(iii) *Differentiation of the Golgi apparatus.* The purpose of this step is to oxidize the silver attached to structures other than the Golgi apparatus. When this is done, the Golgi apparatus stands out clearly because the background is very nearly transparent. At first we bleached the sections in iodine, exactly as recommended by Adamstone (1952). This was not satisfactory since the sections bleached irregularly and it was difficult subsequently to remove all of the silver iodide formed in this reaction. Better results were obtained with a reagent introduced by Farmer (1883, 1884) for bleaching over-exposed photographic negatives. Unfortunately, Farmer called this a 'convenient reducing agent': the only thing that is reduced is the intensity of the photographic image; the silver is oxidized. Farmer's reagent is a 0·3% solution of potassium ferricyanide ($K_3Fe(CN)_6$) in 5% sodium thiosulphate. Silver is oxidized to silver ferrocyanide ($Ag_4Fe(CN)_6.H_2O$), and passes into solution as an argyrothiosulphate complex. The ionic reactions are:

$$\begin{aligned} Fe^{+++}+Ag &\rightleftharpoons Ag^{+}+Fe^{++} \\ Ag^{+}+S_2O_3^{=} &\rightleftharpoons AgS_2O_3^{-} \\ AgS_2O_3^{-}+S_2O_3^{=} &\rightleftharpoons Ag(S_2O_3)_2^{\equiv} \end{aligned} \tag{8}$$

Photographs and sections oxidized in ordinary Farmer's reagent tend to be yellowish. This is due to the deposition of a small quantity of silver sulphide (Ag_2S) from the unstable higher argyrothiosulphate complexes (Lumière and others, 1912; Lüppo-Cramer, 1912). The deposition of sulphide can be prevented by treating the sections for a minute or two in a solution of 1% potassium iodide (KI) in 5% sodium thiosulphate after silvering, and before bleaching (Reindorp, 1935). Farmer's reagent is unstable. When freshly prepared, it is pale yellow-green; after a few hours, it becomes light blue. Ferricyanide is reduced to ferrocyanide by the thiosulphate ion, thus:

$$\underset{\text{(thiosulphate)}}{2S_2O_3^{=}}+\underset{\text{(ferricyanide)}}{2(Fe(CN)_6)^{\equiv}} \rightleftharpoons \underset{\text{(tetrathionate)}}{S_4O_6^{=}}+\underset{\text{(ferrocyanide)}}{2(Fe(CN)_6)^{\text{≣}}} \tag{9}$$

For this reason, the working solution of Farmer's reagent should be prepared immediately before use. Stock solutions of potassium ferricyanide and of sodium thiosulphate, however, are quite stable.

To oxidize unwanted silver, put a slide in a jar of Farmer's reagent, after treating it with iodide/thiosulphate. Watch carefully. The silver attached to the collodion is removed first. That attached to tissue structures is only dissolved after the collodion appears quite free of silver. Once the silver within the sections begins to dissolve, it is advisable to watch the rest of the differentiation under the microscope. The slide should be dipped in a jar of Farmer's reagent from time to time to replace the exhausted ferricyanide and to remove the reaction-products. When the contrast between the Golgi apparatus and the remainder of the tissue is adequate, stop the differentiation by washing under running water (the direct jet of tap-water is adequate) for several minutes.

(iv) *Finishing procedures*. If it is not intended to keep the slide permanently, or if this is later to be bleached and stained, the slide should be mounted now. To do this, rinse in distilled water, and dehydrate in ascending concentrations of ethanol. One can mount directly from absolute ethanol in Gilson's (1906) euparal. This has a conveniently low refractive index, and will, in time, dissolve the collodion membrane. Alternatively, clear in xylene and mount in a synthetic resin, such as DPX (Kirkpatrick and Lendrum, 1939) or the 'synthetic neutral mountant' sold by Flatters and Garnett (309 Oxford Road, Manchester, 13). Canada balsam is not suitable. Even when mounted in the synthetic media, the preparations are not permanent: in time, the silver goes into colloidal solution, especially if subjected to bright illumination.

If it is intended to keep the slide permanently, it is advisable to replace th silver with gold ('gold toning'). Gold toning was introduced more than century ago as a means of rendering daguerreotypes permanent. Gold, bein a 'nobler' metal than silver, will replace silver, exactly as silver would replac copper, or copper would replace zinc. Gold, being very unreactive, produce a permanent image. Proceed as follows: rinse the slide in distilled water an place it in a jar of 0·2% yellow gold chloride ($AuCl_3.NaCl.2H_2O$). Afte 5 to 10 min, rinse in distilled water and leave for a minute or two in 5% sodiu thiosulphate to remove any silver remaining in the sections. Wash unde running tap-water; dehydrate and mount as before. However, Canada balsa can now be used as a mounting medium. Thick sections can be made to appea thinner (see fig. 1) by mounting them in one of the media of high refractiv index intended for mounting diatoms (for instance, the medium of r.i. 1·66 sold by G. T. Gurr as 'Clearax').

Some Causes of Failure

All methods of silvering the Golgi apparatus are somewhat capricious. B this it is meant that sometimes, for no apparent reason, the Golgi apparatu is not adequately blackened or does not stand out in sufficient contrast to b profitably studied. The method of silvering recommended here is no exception. But, as the sections can be examined before, during, and after silvering and as the silvering can be repeated on different sections of the same piece tissue, it is sometimes possible to ascertain the cause of failure.

Not all neuronal perikarya, after fixation in any of the reagents that we hav tried, enclose cytoplasmic objects that resemble those described by Golg (1898). Some enclose only a dense mass of much smaller objects, similar t those described by Flemming (1882). At present it is impossible to sa whether these cells never had a Golgi apparatus, or whether the Golgi apparatus was destroyed by the fixation and the after-treatments. Where ther is no Golgi apparatus before silvering, no Golgi apparatus can be blackene In cases of failure, this possibility should be investigated; for this purpos the interference microscope is invaluable.

Sometimes the Golgi apparatus is blackened to a greater extent in som parts of a section than in others. This is usually due to variations in the thickness of the collodion membrane. Considerable practice is required before th membrane is successfully produced on every slide. Practice is also require before one develops the knack of stopping the differentiation of the Golg apparatus at the right moment. Once the differentiation of the tissue constituents begins, things happen rather quickly: one should have all th reagents required close to hand, and one must act without hesitation.

An occasional complication is the presence of a heavy granular deposit o the sections, usually as concentric rings, up to about 2 mm in diameter. Thi may be due to uneven flooding with the silvering solution, to an insufficie quantity of the reducing fluid, or to delays in washing off the reaction product of the reduction. The remedies suggest themselves. Finally, as in all method of silvering, extreme cleanliness is required: grains of dust are just as argyrophil as tissue constituents.

When neurones were photographed to scale before and after silvering (as was done in fig. 2), it became obvious that silvering produced considerable shrinkage, and that all parts of a neurone did not shrink at the same rate. The cytoplasm shrank more than the nucleus. It is strange that firmly mounted sections could be made to shrink. Most of the shrinkage occurs during oxidation in Farmer's reagent.

DISCUSSION

At first sight it is strange that the Golgi apparatus should have resisted fixation in 'FAM' and the after-treatments required to produce double-embedded sections. Acetic acid is generally supposed to destroy the Golgi apparatus, and ever since Nussbaum's investigations (1913), it has been assumed by many that lipids must be preserved. Baker (1957*a*) has recently demonstrated that acetic acid does not necessarily destroy cytoplasmic inclusions whether these are mitochondria or the Golgi apparatus. Concerning lipids, the situation is less clear. Organic solvents do not readily extract lipids from pieces of tissue that were fixed in formaldehyde (Baker, 1944). Treatment of formaldehyde-fixed material with organic solvents has the further effect of 'unmasking' certain lipids (i.e. splitting from any protein conjugated with them) (Ciaccio, 1926; Clayton, 1958), so that these now become more reactive, even if present in smaller quantities. 'FAM' preserves a surprising amount of lipid, though this fixative should not be used for histochemical studies of the distribution of lipids. If the permeability-hypothesis (p. 210) is correct, the preservation of different substances in the tissue, when it is wished to silver the Golgi apparatus, is important only in that enough of the substances contained in this structure must be preserved for the structure to retain its characteristic shape. Silvering, particularly as done here, cannot be considered a histochemical test for the demonstration of any particular substance.

Do the classical Golgi methods, in which whole pieces of tissue are silvered before embedding, also depend upon the relative permeability of different tissue constituents, rather than upon a chemical reaction between the Golgi apparatus and the silver? There is no proof of this, but the available evidence indicates that this may be so. For instance, in Aoyama's method (1929), small pieces of tissue are fixed in formaldehyde / cadmium chloride for 4 to 12 h; after a brief rinse in distilled water, they are 'impregnated' in silver nitrate for 13 to 17 h. Some reduction takes place during this period, since the tissue contains, at all times, a mixture of formaldehyde and silver nitrate in the presence of cadmium and chloride ions. The silver is reduced very slowly and then only to a colloidal state, presumably because the tissue acts as a protective colloid and because the pH of the mixture in the tissue is not conducive to rapid reduction. Obviously, a certain amount of 'impregnation' occurs at the same time, but it is difficult to say how much. Then, after another rinse in distilled water, the tissue is put in a hydroquinone / formaldehyde / sodium sulphite reducer for about 5 h. It seems probable that this leads to the further reduction of silver upon those sites in the tissue that already contain some colloidal silver after the treatment with silver nitrate. This step would correspond to the repeated silvering and reduction in the method described here. Since in Aoyama's and similar methods some impregnation is unavoidable, and since it is not easy to control each step, inexplicable failures are not uncommon. There remains to be explained the so-called 'adjuvants', such as the salts of cadmium, cobalt, uranium, &c. We have shown that they do not directly influence the preservation of the Golgi apparatus or the ease with which this can be silvered in paraffin sections. Yet the classical methods definitely do not work without 'adjuvants'. Two explanations seem reasonable. Colloidal silver can be precipitated or 'salted out' by the addition of small quantities of salts of certain metals (e.g. lithium, sodium, potassium). However, salts of heavy metals, such as cobalt, cadmium, and uranium, behave abnormally in this respect: they protect the colloid rather than precipitate it (see Ostwald, 1919). This might help to produce particles of silver of the correct size for entering structures that have the permeability of the Golgi apparatus. The second possibility is this: that the meshes of the fixed tissue perform the same function as the collodion membrane in our method, that is to say, form a protective colloid round the particles of silver. The size of the particles that do penetrate the tissue would depend upon the size of the meshes. Since salts such as cadmium chloride, *when used with the fixative*, are powerful coagulants of protein (Baker, 1944, 1950), they would affect the size of the meshes, and therefore of the particles of silver.

Concerning the applications of the method described here, certain limitations should be taken into account. If a certain type of cell encloses a cytoplasmic network, this can almost certainly be blackened by our method. The structure of the deposit of silver then resembles very closely the cytoplasmic network of the living cell. The use of the term 'Golgi apparatus' to denote this type of network is unexceptionable (Cain, 1954). However, objects of various shapes are also blackened by our method (and other Golgi methods) in cells that do not contain similar objects when in the living state. For instance, in the cytoplasm of invertebrate neurones, objects known as dictyosomes are readily blackened: these are the distorted remnants of what, in the living neurones, are separate spherical globules of hydrated phospholipid (Chou, 1957*a*, *b*; Chou and Meek, 1958). The use of the term Golgi apparatus to denote separate lipid globules is clearly unjustifiable. We do not believe that all the objects blackened by our technique should be homologized, unless evidence can be found that they all have approximately the same structure in the living cells, contain the same or approximately the same substances, develop in approximately the same way, and perform the same type of function (see Baker, 1957*b*).

Malhotra (1959) has recently brought new evidence in favour of Legendre's hypothesis (Legendre, 1910) that the objects coloured by basic dyes in the cytoplasm of vertebrate neurones (Nissl, 1885, 1888, 1894), and the Golgi apparatus, were one. When Malhotra silvered the Golgi apparatus of vertebrate neurones, and, after bleaching it, stained the same cells with a basic dye, the structures that had been silvered now took up the dye. He proposed that these structures be called the 'Golgi–Nissl complex'. One possible objection to this theory is that the silver used to blacken the Golgi apparatus and the iodine used to bleach it might have affected the tissue in such a way that the basic dye would become attached selectively to those structures that, before, had contained silver. With the new technique, Malhotra's evidence can be re-examined by performing the experiment the other way round. We have performed this and allied experiments, and hope to describe them in full in a subsequent paper (David, Mallion, and Brown, 1960). Some of the work described here (especially fig. 2) supports Malhotra's thesis. When fixed sections are examined with the interference miscroscope before silvering the Golgi apparatus, and with direct illumination after silvering (see fig. 2), it becomes obvious that no structure of sufficient size or of the right shape to be the objects described by Nissl, remains unsilvered. As our unsilvered sections take up basic dyes in the usual way, it follows that only one type of structure is involved in the silvering and the dyeing. Therefore, Malhotra's hypothesis can be accepted as a basis for further experimental work.

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APPENDIX

The reagents used in techniques of silvering should be carefully prepared and stored. All chemicals should, if possible, be of analytical quality. Below are given the special solutions required. Unless otherwise indicated, they should be weight/volume solutions, i.e. they should contain x g of solute per y ml of finished solution.

Collodion solution

Absolute ethanol	250 ml
Diethyl ether	250 ml; add
'Low-viscosity nitrocellulose' (G. T. Gurr)	5 g

Keep in a well-stoppered bottle.

Silvering fluid

Stock solution: 17% aqueous silver nitrate (about 1 M $AgNO_3$). Immediately before use dilute 25 ml of this with 25 ml absolute methanol.

Reducer

Sodium tetraborate, $Na_2B_4O_7 . 10H_2O$	5·1 g
Undiluted commercial formaldehyde solution	200 ml
Distilled water	to make 500 ml

Farmer's reagent (unstable)

10% aqueous potassium ferricyanide $K_3Fe(CN)_6$	1·5 ml
5% aqueous sodium thiosulphate, $Na_2S_2O_3$	50 ml

Sulphide inhibitor

2% aqueous potassium iodide, KI	25 ml
5% aqueous sodium thiosulphate, $Na_2S_2O_3$	25 ml

Gold chloride

0·2% aqueous yellow gold chloride, $AuCl_3 . NaCl . 2H_2O$

Sodium thiosulphate

5% aqueous sodium thiosulphate, $Na_2S_2O_3$

The Total Solids in the *A*- and *I*-Band Regions of Living Mammalian Striated Muscle-fibres

By K. F. A. ROSS

(*Banting and Best Department of Medical Research, University of Toronto; present address, Zoölogisch Laboratorium, University of Leiden, Kaiserstraat 63, Leiden, Holland*)

and W. G. B. CASSELMAN

(*Banting and Best Department of Medical Research, University of Toronto; present address, Division of Cell Biology, Institute for Muscle Diseases, 515 East 71st Street, New York 28, N.Y., U.S.A.*)

With two plates (figs. 1 and 3)

SUMMARY

Living muscle-fibres from freshly killed mice were mounted in isotonic saline / protein media and examined with a Smith interference microscope, usually with a white light-source. When the *A* or *I* bands near the edge of a fibre were observed to match the colour of the background field, their refractive indices were close to that of the mounting medium; although it is extremely probable that diffraction at the edges of the adjacent unmatched bands affected their apparent match, so that they were not exactly of this refractive index. Matched *A* bands were distinguished from matched *I* bands by examining them in plane-polarized light, by rotating the preparation through a right angle under the interference microscope to display their birefringence, and from the colour of the unmatched bands when the fringe system of the microscope was left unaltered.

In any one fibre, the refractive indices of the *A*-band regions were always higher than that of the *I*-band regions. The *H* bands had lower refractive indices than the *A* bands, and the *Z* bands higher than the *I* bands, but these were both too narrow to be matched satisfactorily by this method. The refractive indices of the solutions in which matched *I* bands were found ranged from 1·358 to 1·363, and those in which matched *A* bands were found from 1·360 to 1·366. The mean refractive index of the *A* and *I* bands was very close to 1·363, which is equivalent to a total solid content of 16% w/v.

These findings are in good general agreement with those of H. Huxley and Hanson (1957) and Bennett (1955), who measured the distribution of solid material in isolated glycerinated mammalian myofibrils; but the difference between the refractive indices of the *A*- and *I*-band regions of the living fibres appeared to be very much less. Only part of this discrepancy can be accounted for by the presence of non-fibrillar solid material, because the total amount of this is extremely unlikely to exceed 50% of the total myofibrillar fibrous protein. It therefore seems probable that, because of the diffraction from the unmatched bands, the true refractive indices of the *A* bands were higher than those of the solutions in which they appeared matched, and those of the *I* bands were correspondingly lower than those of the solutions in which they appeared matched.

The maximum error involved here (when the sarcomere interval was approximately 2·5 μ) can be quantified from independent estimations of the non-fibrillar material in whole muscle (Szent-Györgyi and others, 1955; Hanson and H. Huxley, 1957); and from this it seems highly probable that the refractive indices of the *I* bands were not lower than 1·350 (equivalent to a solid content of 9% w/v), and those of the *A* bands were not higher than 1·375 (equivalent to a solid content of 23% w/v).

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INTRODUCTION

THE development, in the last 8 years, of interference microscopes capable of detecting and measuring very small phase differences, together with immersion techniques for the refractometry of living cells, has enabled very precise measurements to be made of the solid concentrations of the cytoplasm and inclusions in many living cells.

Hitherto, however, these techniques have not been applied to the problem of measuring individually the total solids in the different regions of living mammalian striated muscle-fibres, although A. Huxley and Niedergerke (1958) have obtained values for the refractive index of the *A*- and *I*-band regions of living striated muscles in the frog in the course of their notable studies (by interference microscopy) of their contraction, and H. Huxley and Hanson (1957) have made similar measurements on the different regions of glycerinated mammalian myofibrils.

There are two major difficulties in an investigation of this kind, both arising from the nature of mammalian muscle. First, the tissue itself is very fragile, and it is extremely difficult to isolate and separate single fibres without damaging them at some point along their length, and it is of first importance to ensure that the segment of the muscle on which measurements are being made is in a completely viable condition. Secondly, the very close spacing of the striations introduces optical artifacts and errors in the measurement of phase differences by interference microscopy. The investigation described below was undertaken with the aim of meeting these difficulties and obtaining fairly precise values for the refractive indices (and hence the dry solid content) of the different regions that are distinguishable in visible light in the normal striated muscle of the mouse.

The measurements were made by the matching method of immersion refractometry developed by Barer and Ross in 1952, and fully described by Barer and Joseph (1954, 1955), except that the material was examined by interference microscopy and not by phase contrast. Isolated fibres were mounted in a range of buffered isotonic protein solutions of different refractive indices until one was found in which the *I*- or *A*-band regions of the fibre exactly matched the background field in colour if white light was used (fig. 1), or in intensity if nearly monochromatic mercury light was used (fig. 3). The refractive index of the matched region was then close to that of the mounting medium. These values could then be interpreted in terms of water and solid content, since, as was pointed out by Davies and Wilkins (1951, 1952) and by Barer (1952), the specific refraction increments of nearly all proteins and amino-acids, the principal constituents of cytoplasm and muscle-fibres, are very close to 0·0018. Thus, values for the dry solid content of the *I*-band regions could be obtained to within 1% of solid, and nearly as accurate values could be obtained for the weakly birefringent *A*-band regions.

An interference microscope was used instead of a phase contrast microscope for these measurements, for two reasons.

(1) With phase contrast the 'halo' artifact, inherent in the optical system, that surrounds the image of all unmatched objects, made it impossible, because of the close spacing of the striations, to obtain a reliable match for any of the individual regions, while with the interference microscope this particular optical artifact could be eliminated.

(2) By using the interference microscope with a white light-source, it was possible to obtain valuable supporting evidence for distinguishing the *A*- and *I*-band regions from the colour of the unmatched bands relative to the background.

MATERIAL AND METHOD

Material

For nearly all the experiments described below, the material used consisted of fibres from the gastrocnemius muscle of a freshly killed mouse (*Mus musculus*), although in the first few experiments the pectoralis major muscle was also used with entirely comparable results. Both adult and immature mice were used in approximately equal numbers without any difference being found in their muscle-fibres, and fibres from mice of different laboratory stocks were similarly indistinguishable.

Preparation of the specimen

At first, considerable difficulty was found in isolating the fibres in a viable condition for microscopical examination. Attempts were made to emulate the extremely elegant and delicate preparations of single fibres dissected out along their entire length that A. Huxley and his collaborators have made from the semitendinosus muscle of the frog, *Rana temporaria* (Huxley and Niedergerke, 1954; Huxley and Taylor, 1955), but it was found that in the time necessary to do this (frequently several hours), the mammalian fibres at room temperature invariably deteriorated completely, and a quicker method had to be devised. However, when a portion of a muscle was very rapidly removed from a freshly killed mouse and mounted (in saline or a suitable protein medium) and examined within 5 min of the death of the animal, such preparations were found to contain, together with many obviously damaged fibres, some that appeared in every way intact over several millimetres of their length. Occasionally such fibres occurred at the edge of such a preparation in a position adjacent to a suitable background reference area, so that they could be measured interferometrically, and a few of the initial measurements recorded here were made on fibres prepared in this manner by Dr. Eva Cairns. It was, however, a chancy method, and a much more reliable modification of this technique was later devised by Dr. J. Logothetopoulos, which will now be described in detail.

No anaesthetic was used to kill the mice lest it should affect the tissues. Instead, they were first stunned by a blow on the head and immediately killed by separating the neck vertebrae. The whole gastrocnemius muscle of one leg was then rapidly removed and place in a Petri dish of ice-cold 0·9% saline.

A portion of the belly of the muscle about $\frac{3}{4}$ cm long and 2 or 3 mm wide was then removed by means of a clean cut with fine dissecting scissors in a direction parallel to the line of the fibres (fig. 2, A). The ends of this portion were then cut off squarely (fig. 2, B), and the remaining rectangular piece was gently separated along the natural cleavage lines between the fibres into several smaller bundles about $\frac{1}{2}$ mm in width by means of fine dissecting needles (fig. 2, C). Still smaller bundles, each containing only a very few fibres or single fibres, were then separated out in a similar manner at one end of each of these pieces of tissue and spread out fanwise with the needles (fig. 2, D). The pieces thus prepared were immediately transferred to an excess of their mounting medium in a watch-glass and from there to a drop of the mounting medium on a slide, after which they were covered with a coverslip and at once examined. With a certain amount of practice, such preparations could be made in less than 4 min from the time of the death of the mouse; they contained a very high proportion of apparently undamaged lengths of individual fibres. In fact, out of approximately 50 preparations so made, less than half a dozen contained no intact fibres at the edge of the preparation suitably placed for making interferometric measurements, and all of them contained intact fibres in some place. It was found that the important thing was to avoid excess

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FIG. 1 (plate). Colour-photomicrographs of segments of living muscle-fibres under a Smith interference microscope with a tungsten light source without a filter. All the photomicrographs were taken with a 2-mm shearing objective and are to the same scale, indicated at the bottom of the page. The reference area in every case is on the left of the photograph and the fibres are all orientated with their long axes in the north–south direction in the field.

A, a fibre mounted in a saline / protein solution with a refractive index of 1·360, with the analyser set to give a yellow background. The *I* bands near the edge of the fibre show the same colour as the background.

B, the same preparation as in A, with the analyser set to give a red background. The *I* bands again match the background colour.

C, a similar preparation to that in A and B, mounted in a saline / protein solution with a refractive index of 1·360. The analyser is set to give a blue background and the *I* bands at the edge of the fibre match this colour. The unmatched *A* bands show up in yellow-green, which indicates a shift up the Newtonian series.

D, the same preparation as in C with the analyser set so that the background and matched *I* bands show up in red. The unmatched *A* bands are now blue, which indicates a similar colour shift.

E, a preparation of a fibre mounted in a saline / protein solution with a refractive index of 1·365. The fringe adjustment of the microscope condenser was the same as in C and D. The analyser was set to give a blue background and the *A* bands match this colour. The unmatched *I* bands show up in indigo, which indicates a shift down the Newtonian series, and a phase-shift in the opposite direction to that of the unmatched bands in C.

F, the same fibre as in E, with the analyser set so that the background and matched *A* bands show up in purple-red. The unmatched *I* bands are orange-yellow, which indicates a phase shift in the same direction as in E.

G, a fibre mounted in a saline / protein solution with a refractive index of 1·360. The muscle-bands are exactly aligned with the optical axis of the microscope, so that the *I* bands match the red background colour in the centre as well as at the edge of the fibre.

H, a much-stretched fibre, mounted in a saline / protein solution with a refractive index of 1·360. In the regions of greatest stretch the *I* bands do not match the red background colour but are yellow, which indicates that they have a lower refractive index than the mounting medium.

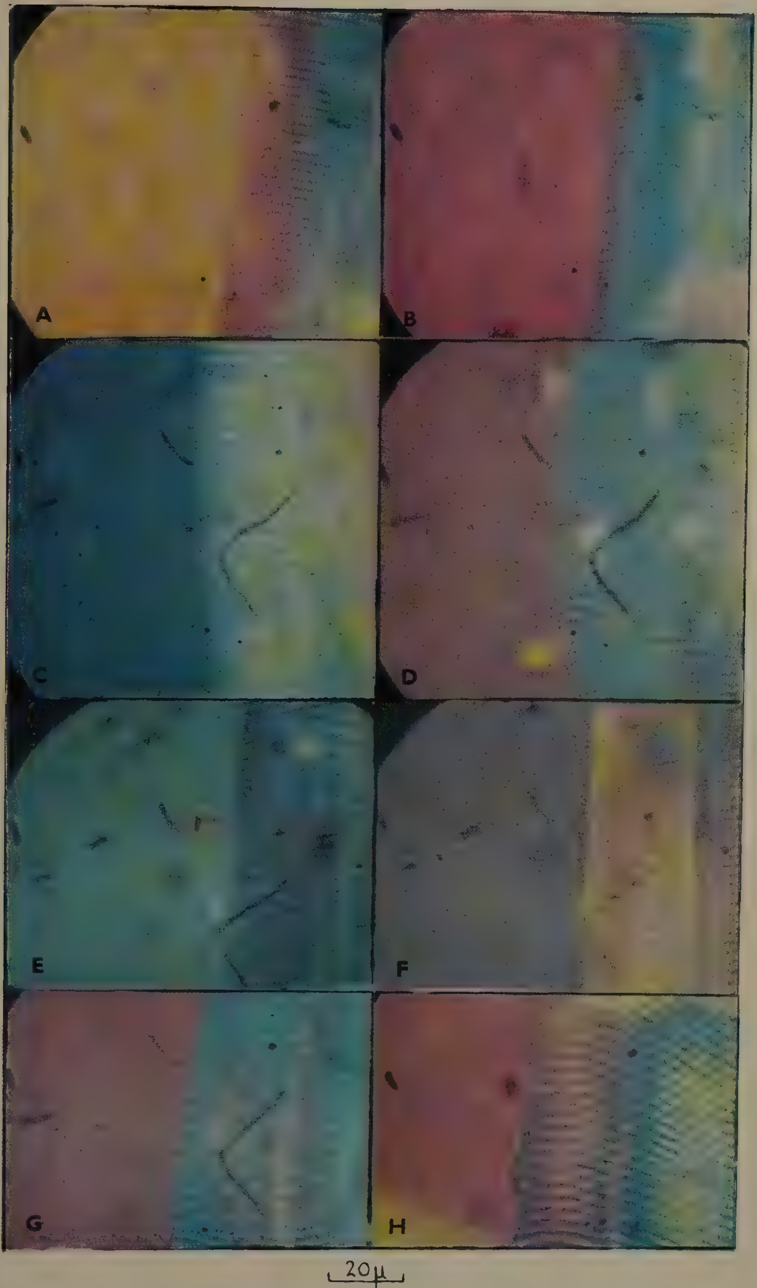

FIG. 1

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teasing, because very thoroughly separated fibres were usually damaged, and it was frequently possible to make completely valid observations on an edge of a fibre that was still in contact with its neighbours (fig. 3, B, C).

It was, of course, most unlikely that any of the fibres so prepared were in fact intact over their entire length, but, for reasons discussed below (p. 234), it seems probable that the portions on which observations were made were in a viable physiological state at the time of their preparation and for a short time afterwards. All the measurements and photographs here recorded were made without delay, and never later than 6 min after the death of the mouse, although the fibres themselves frequently showed no visible signs of necrotic changes for 20 min or longer. The final deterioration of a fibre was often preceded by its undergoing a slow contraction or exuding blebs of homogeneous material on its surface, after which the striations usually became faint or disappeared, and irregular areas of different phase-change spread across the preparation.

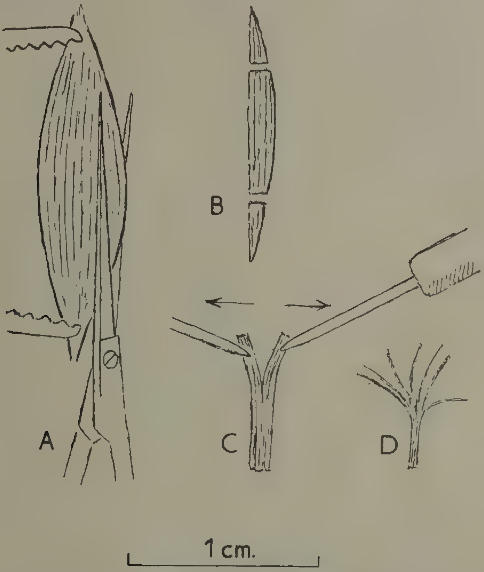

FIG. 2. Illustrations of the rapid dissection technique developed by Dr. J. Logothetopoulos for making viable preparations of muscle fibres. A, a portion being cut from the belly of the muscle. B, the same portion with its ends cut off, leaving an approximately cylindrical bundle of parallel fibres. C, this bundle being separated by dissecting needles into smaller bundles about ½ mm in diameter. D, the final preparation ready for mounting: one of the small bundles 'fanned out' by further gentle separation with dissecting needles into bundles of only a few fibres or single fibres. Stages A–C were performed in a Petri dish of ice-cold saline. Stage D was done on the microscope slide in a large drop of the protein / saline mounting medium. The processes illustrated took about 2 min to perform. (Redrawn from a rough sketch by Dr. J. Logothetopoulos.)

Preparation of the mounting media

The protein solutions used as mounting media in order to match the refractive indices of the different regions of the muscle-fibres were made from Armour's bovine plasma albumin, fraction V, dissolved in saline. The dried albumin powder contains a small amount of salt, and also itself ionizes in aqueous solution. Ross, in 1952, studied its effect on the shrinkage and swelling of spherical cells and concluded that its solutions exert an osmotic pressure equivalent to a dry salt content of $\frac{1}{2}$ to 1%. Thus a 40% solution in distilled water had a tonicity approximately equal to that of a 0·3% NaCl solution, and a solution of this concentration isotonic with mammalian blood (0·9% NaCl) could be made by dissolving the powder in a 0·6% NaCl solution, and this could be diluted with 0·9% NaCl to any concentration required. All these solutions, however, were acid (about pH 5·0), and although this does not appear to affect many living cells when these media are used for immersion refractometry, it was found that they frequently caused the muscle-fibres to go into a state of tonic super-contraction. Consequently a solution of sodium bicarbonate was used as a buffer, and a 40% solution of the bovine plasma albumin in a 0·6% NaCl was diluted with a 1·3% $NaHCO_3$ solution, which has a tonicity equivalent to that of a 0·9% NaCl solution. At dilutions below 20% of protein, such as were suitable for the immersion refractometry of the muscle material, these solutions had a pH between 6·8 and 7·2 (by Beckman meter), and in them the fibres nearly always appeared relaxed and susceptible to measurement. Thus the tonicity and pH of the immersion media were adjusted to approximate to physiological conditions. The refractive indices of the solutions, which ranged from 1·35 to 1·37, were all measured with a

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FIG. 3 (plate). Photomicrographs of segments of living muscle-fibres under a Smith interference microscope, with a tungsten light-source and an Ilford 807 mercury-green filter. A–C under a 4-mm shearing objective, scale as in B. D–E under a 2-mm double focus objective, scale as in D.

A, a fibre, 75 μ in width along the line *a–b*, mounted in saline. The analyser set to give maximum background extinction. Four dark fringes are visible between the edge and middle of the fibre. This indicates a phase change of approximately 4 wavelengths in the light passing through its thickest part.

B, a similar fibre mounted in a saline protein medium of refractive index 1·360 with the analyser set as before. One set of bands matches the background field.

C, the same preparation as in B, with a different analyser setting. The same set of bands matches the background field, now bright.

D, another fibre mounted in a saline / protein medium with a refractive index of 1·360 under a 2-mm double-focus objective, showing the narrow *H* bands. The *I* bands match the background intensity. The *A* bands show up dark, which, at the analyser setting here used, indicates that they have a higher refractive index than the *I* bands. The *H* bands are paler than the *A* bands. This indicates a lower refractive index than the *A* bands.

E, a similar fibre under the same objective mounted in a saline / protein medium with a refractive index of 1·364. The *A* bands of the fibre, orientated with its long axis in the north–south direction in the microscope field, match the background. This indicates that the lower of these two refractive indices was equal to that of the mounting medium.

F, the same region of the fibre shown in E, orientated in the east–west direction in the microscope field. The *A* bands no longer match the background, which clearly demonstrates their anisotropic nature.

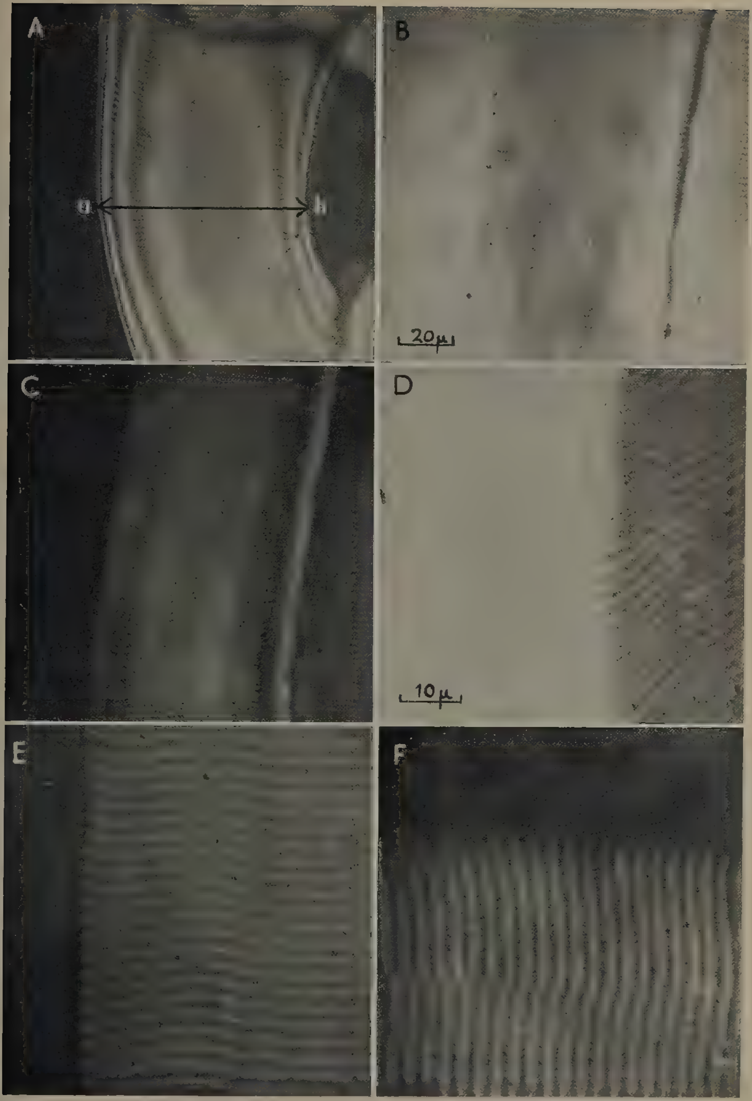

FIG. 3

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Bellingham and Stanley pocket refractometer, with a built-in yellow filter with a transmission equivalent to the mean of the two sodium lines (589 mμ).

Microscopic examination and orientation of the specimens

The fact that striated muscle-fibres contain *A*-band regions that are appreciably birefringent means that the matching of such regions with the background field depends not only on the refractive index of the mounting medium, but also on the plane of vibration of the light passing through them. This, at first sight, would suggest that an interference microscope that does not itself use a polarizing optical system, such as the Dyson interference microscope manufactured by Cooke, Troughton, and Simms of York, would be better for making measurements on a material of this kind, because it would be possible to measure both the refractive indices of these anisotropic regions by the introduction and orientation of a suitable polarizer. A Dyson microscope was in fact used with some success on one occasion. However, it is a difficult instrument to set up and adjust very rapidly, and the need for speed in getting the specimens ready for examination necessitated the use of an instrument that was simpler to operate, and the Smith double-refracting interference microscope was found to be considerably more suitable.

Two Smith interference microscopes were used in the course of the work, one manufactured by Messrs. Charles Baker of Holborn (the Baker interference microscope) and one manufactured, under licence, by the American Optical Co. of Buffalo (the A. O. Baker interference microscope). The latter had a rotating stage for the easy orientation of the specimen, while the former was capable of being rapidly converted into a plane-polarizing microscope for distinguishing the *A* and *I* bands. Although both instruments thus possessed different advantages, they were optically identical and quite capable of measuring the different refractive indices of the birefringent *A*-band regions when the fibres were correctly orientated in the microscope field. This is because their condensers and objectives are fixed so that the plane of vibration (electric vector) of the 'ordinary' object beam is in the 'north–south' direction of the microscope field (as normally viewed by an observer from behind the instrument) in the case of all the 'shearing' objectives and the 16 mm and 4 mm 'double-focus' objectives; and in the 'east–west' position in the case of the 2-mm 'double-focus' objective (Smith, 1958).

In striated muscle, the plane of vibration (electric vector) of the faster ordinary ray in the (positively birefringent) *A*-band regions is in the direction at right angles to the long axis of the fibre, so that if the fibre is orientated in the 'north–south' direction in the field and observed with any objective other than the 2-mm double-focus, it is possible to measure the *higher* of the two refractive indices of this region. Conversely, with the 2-mm double-focus objective, a fibre so orientated would enable the *lower* of the two refractive indices to be measured. In practice, it was hardly necessary to measure both refractive indices, since the birefringence of the *A*-band regions is actually very small, and the difference is barely within the limits of experimental accuracy when translated in terms of water and solid content (see p. 238). Nearly all the measurements were made with 2-mm and 4-mm shearing objectives, and this necessitated a north–south orientation of the fibres because the east–west direction of the shearing systems would otherwise give overlapping images. Thus, most of the measurements made on the *A*-band regions were of the higher of its two refractive indices. The *I*-band regions, of course, presented none of these difficulties, and if matched to the background, remained so in whatever direction they were orientated.

MEASUREMENTS

Preliminary measurements

Approximate values for the refractive index of the striated muscle material were obtained from the examination, by interference microscopy, of isolated single fibres mounted in 0·9% NaCl, because these were of sufficient thickness to retard the light passing through them by several wavelengths when they were mounted in a medium with a refractive index close to that of pure water, and this retardation was visible in the form of a series of interference fringes. One such fibre which had a diameter (measured with an eyepiece micrometer) of 52 μ, when viewed in nearly monochromatic light (obtained by using an Ilford 807 mercury green gelatine filter with a tungsten light-source), showed a total of 3 dark longitudinal fringes across the width of the fibre in both the *A*- and *I*-band regions between its edge and middle line. This indicated a phase retardation of approximately 3 wavelengths through its thickest part; and this meant that, if the thickness of the fibre in the direction of the optical axis of the microscope was equal to its width at right angles to this, an approximate value for the refractive indices of both the *A*- and *I*-band regions, n, could be obtained from the formula

$$n = \left(\phi \times \frac{\lambda}{t}\right) + m,$$

where ϕ = the phase change in the middle of the fibre in wavelengths (about 3), λ = the mean wavelength of the light used (0·54 μ approximately), t = the measured width of the fibre (52 μ), and m = the refractive index of the saline mounting medium (1·334). This gives an approximate value of 1·366 for the refractive index of the muscle material. Similarly, in another, partly contracted, fibre shown in fig. 3, A, a total of 4 dark bands (alternating with 4 bright bands) can be discerned between the edge and middle line of the fibre along the line *A–B*, where the width of the fibre is 75 μ. This gives an approximate value of 1·363 for its refractive index.

With these data as a guide, similar muscle-fibres were then mounted in a protein medium with a refractive index of 1·360, and it was found that, under the interference microscope, one set of bands did in fact match the background in colour at all settings of the analyser when white light was used, and in intensity when nearly monochromatic green light was used. Fig. 3, B, C shows one such fibre in green light at two different analyser settings; the latter with the intensity of the background field, and matched bands adjusted so as to give maximum extinction. In the course of just over 50 subsequent experiments, matched bands were visible in 40 preparations that had been mounted in media with refractive indices between 1·358 and 1·366. In the remaining preparations, which were mounted in media with refractive indices just above and below this range, no matched bands were seen. This meant that the refractive indices of the *I*- and *A*-band regions all lay close to this range of values; and it was obviously necessary that they should be distinguished from one another and, if possible, measured individually.

Distinguishing the I- and A-band regions

The certain recognition of the *I*- and *A*-band regions in the fibre was not quite as simple as might at first appear, since neither their relative widths (which were usually about equal) nor their appearance in ordinary unpolarized white light provided reliable criteria.

Three reliable methods, however, were found; and all of them were used, at different times, for distinguishing the bands.

The most obvious method was to use the appearance of the different regions in plane-polarized light. When the fibres were placed between crossed polaroids giving background extinction, their *A* bands showed up brilliantly. It was necessary first to find a matched band under the interference microscope that was in some way distinctive or individually recognizable by means of some sort of marker. The condenser of the microscope was then removed and replaced with an ordinary Abbe condenser, and the interference objective was replaced with a Watson 2-mm fluorite objective in another objective-holder. The quarter-wave plate was removed and the polarizer and analyser of the microscope adjusted to give extinction. By this means, matched *I* bands were demonstrated in several preparations mounted in a medium with a refractive index of 1·360; and matched *A* bands were similarly recognized in a medium with a refractive index of 1·365. The disadvantages of this method were that it was not always easy to find a band that was clearly recognizable with both optical systems, and also the necessary delay of about 2 min in changing the lenses and readjusting the microscope involved some risk of the specimen deteriorating.

The second method depended on the fact that the *I*-band regions, being isotropic, will continue to appear matched to the background however the fibre is orientated in the microscope field, while the anisotropic *A* bands, if they should be matched when lying in one position, will not be so when they are turned at right angles to it. Fig. 3, E shows a fibre mounted in a medium with a refractive index of 1·364, and viewed in nearly monochromatic green light with a 2-mm double-focus interference objective. It is orientated with its axis in a 'north–south' direction in the microscope field; and the *A* bands match the intensity of the background field, which means that the lower of their two refractive indices was 1·364. Fig. 3, F shows the same region of the same fibre rotated through a right angle, so that its axis is now east–west; and the *A* bands that were matched are now appreciably darker than the background field.

The main disadvantage of this second method was that it was necessary to use double-focus interference objectives, since only these would permit the rotation of the preparation through a right angle without its being masked by a double image: and the use of these objectives for making measurements on rather large objects like muscle-fibres is open to serious criticism on account of the 'halo' artifact produced by the superimposed out-of-focus secondary image. It is therefore quite possible that matches obtained on the muscle-bands with these objectives are subject to error; although such errors cannot be very large, since all the measurements so made were within the limits of those obtained with the shearing objectives.

The two methods outlined above served to establish (if it were necessary) that the *A*-band regions consistently had slightly higher refractive indices than the *I* bands; and it was then possible to use a simpler, third method to determine which set of bands had been matched in any given medium—from the colour of the unmatched bands when a white light-source was used.

The Smith interference microscope is a flexible instrument and it is possible to adjust it so that, for a given rotation of the analyser, the background field will change colour either up the Newtonian series of interference colours, or down it, depending on the direction of tilt of the condenser surfaces relative to the objective. (This in no way affects the fact that it is always necessary to rotate the analyser in a certain direction in order to make a phase-change measurement, because a phase object in the field will always exhibit a colour shift in the corresponding direction.) Therefore the colour of an unmatched object relative to the background does not in itself give any indication as to whether the object is giving an advance or retardation in phase of the light passing through it. If, however, the initial adjustment of the microscope is left unaltered, and two different specimens are examined in succession, it is possible to distinguish between two phase-advancing or phase-retarding objects, or between objects giving phase changes of different sign, by means of the direction of the shift in colour that they exhibit. If, for example, the first object were known to be a phase-retarding object of higher refractive index than its mounting medium, and it appeared in a colour slightly higher in the Newtonian series than that of the background, and the second object, with the same background colour, had a colour lower in the Newtonian series, it would mean that the second object was giving an acceleration in phase and therefore had a lower refractive index than the mounting medium.

This can be seen in the colour photomicrographs of muscle-fibres shown in fig. 1, C–F. C and D show a fibre mounted in a medium with a refractive index of 1·360, while E and F show a similar fibre mounted in a medium with a refractive index of 1·365, the microscope being left in exactly the same adjustment for all the photographs (and for A, B, G, and H as well). In C and E the analyser has been adjusted so that the background and the matched bands are of a similar light-blue colour, but the unmatched bands in C are yellowish-green, a shift up the Newtonian series, and in E they are dark blue, a shift down the series. Similarly, in D and F the background and the matched bands are of approximately the same purplish-red colour, but in the first case the unmatched bands are blue and in the second case they are yellow. This can only mean that if, in the preparation mounted in the medium with a refractive index of 1·360 (C, D), the unmatched bands were giving a phase retardation and were therefore of higher refractive index, and were *A* bands, then the unmatched bands in the preparation mounted in the medium with a refractive index of 1·365 (E, F) must have been phase-advancing and therefore of lower refractive index, and hence were *I* bands. Therefore, provided that the *A* bands are invariably of higher refractive index than the *I* bands, which seems highly likely, if it was the *I* bands that were matched in the first medium of refractive index 1·360, it must have been the *A* bands that were matched in the other medium of refractive index 1·365.

It would, of course, have been equally possible to determine the sign of the phase-change given by the unmatched bands by using nearly monochromatic light and rotating the analyser in an appropriate direction from the position of background extinction and seeing if they darkened or brightened, but the method described above was simpler and quicker, since it depended on only one observation and the colour of the unmatched bands could be recognized at once.

Results

With the aid of these criteria for distinguishing the *I*- and *A*-band regions, it was possible to measure their refractive indices individually with the following results, based on the examination of just over 50 specimens.

I-band regions were found matched in media having refractive indices ranging from 1·358 and 1·363, but not outside this range. In these preparations, the *A* bands always appeared to give a phase retardation (figs. 1, A–D, G; 3, B–D).

A bands were similarly found matched in media with refractive indices ranging from 1·360 to 1·366. These measurements, however, were made with a 2-mm shearing objective with the fibre orientated north–south, and therefore represent the limits of range found for the higher of the two refractive indices of the *A*-band material. A few measurements of the lower of the two refractive indices of the *A*-band regions were also made with a 2-mm 'double focus' objective with the fibres at the same orientation, but too much reliance was not put on them for reasons already discussed (p. 232). These gave values of 1·364 and 1·365 (figs. 1, E, F; 3, E, F).

The less extensive *Z*-band and *H*-band regions, in the middle of the *I* and *A* bands respectively, were also frequently observed under the interference microscope, but they were too narrow to be matched with certainty. From their colour, however, it was clear that their refractive indices were close to those of the *I*- and *A*-band regions. The *Z* bands always appeared to have a slightly higher refractive index than the *I* bands, and the *H* bands slightly lower than the *A* bands.

Problems of the Experimental Method

The viability of the fibres measured

As already mentioned, measurements were only made on portions of fibres that appeared in every way to be absolutely undamaged, but it was certain that practically all of these had been cut at some point along their length. It could therefore be argued that, being no longer completely intact, none of the portions examined was in a physiological condition comparable to those in the living animal, and that the refractive index measurements made on them were not necessarily the same as for undissected muscle. This argument cannot be entirely refuted, but the results obtained strongly suggest that it is not in fact valid.

If the surface of a muscle-fibre is damaged so as to become permeable to the molecules of the mounting medium, one would expect an interchange of material to occur between the inside and outside, causing gradients of refractive index and phase change; and this is in fact what was found in the case of all the obviously damaged fibres. The cut ends of the apparently intact fibres also nearly always showed similar phase gradients, but it took many minutes for these to spread inwards over a distance of several hundred microns to the regions where the measurements were made. It was also very unlikely that a very rapid and completely uniform alteration in water content (causing an alteration in refractive index) had taken place at the moment that the fibres were dissected, because of the extremely narrow range (1·358 to 1·366) of refractive index found in all the apparently intact fibres measured. One would not expect any considerable alteration of refractive index, if it occurred, to be so uniform; and fibres whose refractive indices had been appreciably changed by handling could reasonably be expected to exhibit a greater degree of variation. The maximum range in refractive index of the material was probably very little greater than that measured, because although most of the fifty-two fibres that were examined were in media within these limits, 12 were in media with refractive indices just outside these limits, and none of these fibres appeared matched, although in other respects they seemed normal.

Occasionally, in a fibre which had been appreciably stretched, parts of otherwise matched *I*-band regions showed a small negative phase change, indicative of a slightly lower refractive index (fig. 1, H). The significance of this observation will be discussed below (p. 236). Nearly all the fibres examined, however, were in a 'resting' condition (unstretched and uncontracted), with a sarcomere interval very close to 2·5 μ.

Optical difficulties involved in matching the muscle-bands

Because the sarcomere interval (about 2·5 μ) was small compared to the total thickness of a fibre, it was only on rare occasions that one could find a preparation where the *A* and *I* bands were exactly aligned in the direction of the optical axis of the microscope through the whole thickness of the fibre: but cases of this can be seen in figs. 1, G and 3, B, C, where the matched bands stretch right across the fibre. It was, however, only necessary to obtain a match in the thinner regions 5 to 10 μ in from the edge, and it was of little importance if the bands in the centre were out of alignment.

The thickness of the fibre, however, made measurements even in this region open to one serious objection, for which the writers are indebted to Professor H. S. Bennett of the University of Washington, Seattle. This is that with a relatively large cylindrical object such as a muscle-fibre seen under a microscope objective of high numerical aperture, it is inevitable that some of the more oblique illuminating rays must pass through the under surfaces of the fibre to reach the region tangential to the optical axis on which the measurements were made, and this might introduce errors into the phase-change measurements. Although this was less likely to be important here, when it was in fact zero phase changes that were being measured, it was obviously important to devise some way of determining whether the fibre thickness did have an effect. The experiment described below was suggested by Dr. A. Szent-Györgyi at Woods Hole.

The living fibres were never less than 50 μ in thickness, but fixed preparations of fibres could be cut into much thinner sections. Unstained preparations were therefore made of material fixed in Carnoy's fluid and in formalin, which was embedded in paraffin wax and cut longitudinally into sections varying from 10 μ to 30 μ in thickness. It was then necessary to find a suitable mounting medium of the same refractive index as one of the band regions of this fixed material and, in the 10-μ sections, it was found that in methyl salicylate, with a refractive index of 1·536, one set of bands exactly matched the background in colour and intensity under the interference microscope. The 30-μ sections were then similarly mounted and examined, and exactly the same match was obtained.

Although the maximum thickness of the sections here was only about half that of the living fibres, the experimental conditions were more closely comparable than would at first appear, since the sarcomere in the fixed material was also appreciably less. Therefore, although it proved nothing, this experiment quite strongly suggested that this particular factor was probably not important in the matching of the bands with the living material. Moreover, as was pointed out by A. Huxley (1958), living muscle-fibres are seldom exactly circular in cross-section, but are much more usually polygonal so as to fit snugly against adjacent fibres, and quite often have 'sharp' edges. It is certain that many of the measurements made were through such edges, where there was a lesser thickness of the material.

The small sarcomere interval of the resting fibres that were almost exclusively examined does, however, give rise to another very much more serious optical difficulty, for it means that the individual *A*-band and *I*-band regions were both only about 0·54 μ wide. This means that the centre of each *A* and *I* band was no more than 0·27 μ from the phase boundaries of the adjacent *I* and *H*, and *A* and *Z* bands, respectively. At this distance it is highly probable that any phase-change measurement made in an *A*- or *I*-band region (including the zero phase change indicated by an apparent match) will be affected by diffraction from the images of the adjacent bands. H. Huxley and Hanson (1957), considering this in connexion with their measurements on glycerinated myofibrils, obtained densitometer traces of the diffraction gradients at the vertical edges of a uniform phase object (a myoglobin crystal) with the interference microscope, and concluded that no accurate phase-change measurement could be made less than 0·3 μ away from a phase boundary.

It therefore seems extremely probable that the match obtained by the present method between a muscle-band and the background field did not indicate a zero phase change in this region, but that the *I* bands had somewhat lower refractive indices than those of the solutions in which they appeared matched, and the *A* bands had correspondingly higher refractive indices than the solutions in which they appeared matched. Confirmation of this was rather strikingly provided by the few instances in which stretched fibres were observed mounted in the protein media, because, as already mentioned (p. 234), the *I* bands then appeared to have a lower refractive index than when the muscle was in the resting condition (see fig. 1, H). H. Huxley and Hanson (1957) used stretched myofibrils to obtain valid measurements for the phase changes in the *I*- and *H*-band regions, and they calculated the solid content of the *A*-band regions from the ratio of these measurements; but with the present technique it was not normally possible to stretch the muscle-fibres. Fortunately, however, it is possible to estimate the maximum errors involved in measuring the refractive indices of the *A* and *I* bands of the unstretched fibres from independent measurements of the non-fibrillar material in whole muscle; and this will be discussed in the final section of this paper.

CONCLUSIONS

The most striking components of the *I*- and *A*-band regions of striated muscle are the fibrous protein elements located in the myofibrils. These have recently been shown, mainly through the very careful and complete histochemical and electron-microscopic investigations of H. Huxley and Hanson, to be longitudinally orientated systems of submicroscopic rodlets of actin and myosin that interdigitate and overlap each other in the *A*-band regions (H. Huxley and Hanson, 1954, 1957; H. Huxley, 1953, 1957). In the *I*-band regions only actin rodlets are found, and the *H*-band regions are bridged by the myosin rodlets without actin. Our present results are in complete accord with these findings, because the *A*-band regions were consistently found to have higher refractive indices than the *I* bands or the *H* bands. This is best illustrated in fig. 3, D, where the lighter regions of the fibre, the matched *I* bands and the pale *H* bands, represent the regions of lower refractive index.

The mean refractive index of the *I* and *A* bands found by the present method was 1·363. This is approximately equivalent to a total solid concentration of 16% w/v, or 88% of water assuming that the solid constituents of these regions have a specific volume of 0·75. This indicates a rather higher water content than the generally quoted figure of 80% of water in mammalian muscle, based on weighing and drying. Estimates based on bulk weighing, however, are often rather inaccurate (see, for example, Ross and Billing's findings compared to those of Henry and Friedman with respect to the water content of bacterial spores (Ross and Billing, 1957); and not much reliance should be placed on them. Huxley and Niedergerke's (1958) value of 1·378 for the mean refractive index of the *A* and *I* bands of whole muscle-fibres in the frog is, however, more nearly in agreement with this figure; and it remains a possibility that in the present investigations the refractive indices of the portion of fibre examined were somewhat lowered through some sort of leakage at the cut ends. This, however, seems rather unlikely, for the reasons already discussed on p. 234, and in the argument that follows we shall assume that our value is correct.

The refractive index of the solution in which matched *I* bands were most frequently found was 1·360, and that in which matched *A* bands were most frequently found was 1·365. If these really represented the refractive indices of the *I* and *A* bands, the *A* bands would contain only about 3% more solid matter than the *I* bands. However, H. Huxley and Hanson (1957), in their very thorough quantitative investigation of the fibrous protein components in isolated glycerinated myofibrils from the psoas muscle of the rabbit, found that the *A*-band regions contained approximately 3 times as much solid material per unit length as the *I* bands: an enormous discrepancy. Some of this discrepancy might be accounted for by assuming that the intact fibres contained a considerable amount of uniformly distributed extra material in addition to the fibrous actin and myosin in the myofibrils (in the sarcolemma and other loci); but in order to account for the whole of this difference the amount of this non-fibrous material would have to exceed 10 times that of the fibrous protein in the myofibrils, which is exceedingly unlikely. It is therefore much more probable that most of the discrepancy is accounted for by the diffraction effect already discussed on pp. 235 and 236; and that the true refractive indices of the *A* bands are considerably higher than 1·365, and those of the *I* bands lower than 1·360.

Actually, Szent-Györgyi, Mazia, and Szent-Györgi (1955) and Hanson and Huxley (1957) have made estimations of the non-fibrous protein in whole muscle-fibres, and have found that this does not exceed 50% of the fibrous protein in the myofibrils. Taking Hanson and Huxley's lower figure of 33%, if this non-fibrous protein were uniformly distributed along the length of a fibre it would have a dry mass per unit area of almost exactly 0·6 times that of the fibrous protein (actin) in the *I*-band regions. The *A* bands in the fibres would therefore have 2·25 times as much total solid material per unit length as the *I* bands. Assuming the mean solid content of the *I* and *A* bands to be 16%, if the true solid content of the *I* bands was 10% and the true solid content of the *A* bands 22%, the above *A*:*I* ratio of 2·25:1 would be correct. Because, in the resting fibres examined, the *A*- and *I*-band regions were almost equal in width, it is reasonable to expect that the low error in the measurement of the refractive index of the *A* bands will almost equal the high error in the measurement of the *I* bands. This means that, at its highest, the refractive index of the *A* bands was 1·375 rather than 1·365, or 0·01 higher than that of the mounting medium in which they most frequently appeared matched; and, at the lowest, the refractive index of the *I* bands was 1·350 rather than 1·360, or 0·01 lower than that of the medium in which they most frequently appeared matched.

These represent plus and minus errors of just over 4% w/v for the solid content of the *I* and *A* bands respectively. They are, however, maximum errors based on the assumption that the non-fibrous solid material is uniformly distributed, when in fact it may be more concentrated in the *I*-band regions as suggested by A. Huxley and Niedergerke (1958). It is, however, almost certain that there is an error due to diffraction from the unmatched bands, and that the true refractive indices of the *A* and *I* bands lie somewhere between those of the solutions in which they appeared matched and the upper and lower limits just defined. (The above estimates for the *A* bands, however, are based on the higher of their two refractive indices, and as their birefringence can be taken as approximately 0·004, their mean refractive index can be reckoned as being 0·002 lower, and consequently their solid content 1% less than the figures given.)

It seems probable that the values obtained by Bennett (1955) for glycerinated myofibrils, and the values quoted by Huxley and Niedergerke (1958) for the refractive indices of the *I*-band regions in frog muscle when the sarcomere interval was less than 3 μ (and those of the *A* bands where the sarcomere interval was 3 μ or over), were subject to the same kind of diffraction error as that found here.

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